Transfection of newt blastema mesenchyme using the techniques of lipofection and direct gene transfer

by

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A thesis
submitted to the Department of Biological Sciences
in partial fulfillment of the requirements
for the degree of
Master of Science

September 1992
Brock University
St. Catharines, Ontario

ABSTRACT

The regenerating amphibian limb provides a useful system for studying genes involved in the establishment of positional information. While a number of candidate genes that may play a role in pattern formation have been identified, their function *in vivo* is unknown in this system.

To better ascertain the role of these genes, it would be useful to be able to alter their normal patterns of expression *in vivo* and to assess the effects of this misexpression on limb pattern. In order to achieve this, a method of introducing a plasmid containing the cDNA of a gene of interest into a newt blastema (a growth zone of mesenchymal progenitor cells) is needed. Unfortunately, most commonly used transfection techniques cannot be used with newt blastema cells.

In this study, I have used the techniques of lipofection and direct gene transfer to introduce plasmid DNA containing reporter genes into the cells of a regenerating newt limb. The technique of lipofection was most effective when the blastema cells were transfected in vitro. The optimal ratio for transfection was shown to be 1:3 DNA:Lipofectin (w/w), and an increase in the amount of DNA present in the mixture (1:3 ratio maintained) resulted in a corresponding increase in gene expression.

The technique of direct gene transfer was used to transfect newt blastema cells with and without prior complex formation with Lipofectin. Injection of plasmid DNA alone provided the most

promising results. It was possible to introduce plasmid DNA containing the reporter gene β-galactosidase and achieve significant gene expression in cells associated with the injection site. In the future, it would be interesting to use this technique to inject plasmid DNA containing a gene which may have a role in pattern formation into specific areas of the newt blastema and to analyze the resulting limb pattern that emerges.

ACKNOWLEDGEMENTS

I would like to thank Dr. Carlone for his guidance and encouragement. Also, thanks to Dr. Castle for always being available to help when problems arose in the lab. Also, to my husband and my mother, for being supportive.

I would also like to thank all my friends at Brock for all the good times, and especially to Kirsty Salmon for all her help.

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INTRODUCTION

The phenomenon of limb and tail regeneration is restricted to a few species of amphibians, occuring only in the urodeles and larval anurans. Regeneration is known to begin with the formation of a blastema at the amputation plane, but the mechanisms underlying this process are not well understood (Wallace, 1981). The basic mechanisms of tissue patterning during regeneration appear to be similar to that of development (Muneoka and Bryant, 1982). They are not identical, however. For example, they differ in their dependence on the nervous system for growth control (Fekete *et al*, 1988). Nevertheless, the regenerating limbs of newts and salamanders serve as appropriate models for studying pattern formation in developing limbs of vertebrates.

Many interesting features of the urodele blastema have been uncovered in recent years. For example, the blastema is known to be specified by its location of origin and will regenerate its characteristic structure when transplanted to an ectopic location. Thus, if a forelimb blastema is transplanted to an irradiated tail stump, a forelimb will regenerate (reviewed by Stocum, 1984).

The cells of a urodele blastema possess a "memory" of their origin along the proximal-distal (P-D) axis of the limb (Stocum, 1984). If a limb is amputated at the wrist, a hand regenerates. If a limb is amputated mid-radius and ulna, the missing structures will regenerate to give a complete limb .

The molecular basis for this positional memory is unknown. It is possible however, to alter this positional memory experimentally, either by applying certain chemical agents, or by surgery (Bryant *et al*, 1981; Stocum, 1984). One class of compounds which are able to reset the axial specification of the mesenchymal cells of a limb are the retinoids, in particular retinoic acid (RA). RA is able to affect both the anterior-posterior (A-P) axis in the chick limb (Thaller and Eichelle, 1987), and both the proximal-distal (P-D) axis as well as the transverse axes in urodeles (Thoms and Stocum, 1984).

Some recent discoveries have helped determine how the retinoids are able to produce these effects. It is thought that RA is transported to the nucleus where it binds to retinoic acid receptors (RARs) (Summerbell and Maden, 1990). The DNA-binding domain of the RARs then bind to regulatory sequences of specific genes, leading to their up or down regulation (Summerbell and Maden, 1990). These genes may play important roles in pattern formation in the limb.

It is of great interest to identify and characterize some of the genes which may be involved in specification of pattern in the limb, some of which may be regulated by the retinoids. Currently, many different molecular biological and biochemical techniques are being used to identify these genes. Some of the candidate genes that are being studied with respect to their roles in pattern formation include a number of homeobox genes and retinoic acid receptor genes (Savard *et al*, 1988; Tabin, 1989; Giguere *et al*, 1989; Ragsdale *et al* 1989).

Recently, a number of proteins have been identified which may represent products of genes that play a role in the specification of positional information along the P-D axis of the regenerating limb of the newt *Notophthalamus viridescens* (Carlone *et al*, 1992). Once these genes are cloned, it may then be possible to assess their role in this process. A method for achieving this would be to misexpress their gene products in the cells of a regenerating limb. A technique for introducing the cDNA for the gene of interest into blastema cells is thus required.

Most of the current methods used for transfecting mammalian cells are not appropriate for use with newt blastema cells, for reasons which include toxicity and inefficiency. However, a recently developed technique called lipofection may be successful for transfection of blastema cells *in vivo.* Lipofection has been used to transfect neurons from the embryonic brain of *Xenopus* (Holt *et al*, 1990) as well as blastema cells in culture (Tabin, personal communication). Also, a method involving direct *in vivo* injection of cDNA has recently been shown to be successful with mouse muscle tissue (Wolff *et al*, 1990), and may be an appropriate technique to use for introducing cDNA into newt blastema mesenchyme.

The objectives of my thesis were to:

a) Use the techniques of lipofection to attempt to improve on the reported transfection efficiency *in vitro* of blastema mesenchyme of the newt *Notophthalamus viridescens.* This involved an attempt to optimize transfection conditions by varying the concentration of DNA, varying the ratio of DNA to Lipofectin, and optimization of the duration of transfection with Lipofectin.

b) Use the method of direct *in vivo* injection to introduce plasmid DNA into newt blastema mesenchyme. The DNA was injected with and without prior complex formation with Lipofectin, and the transfection efficiencies using these two methods were compared.

LITERATURE REVIEW

Limb Regeneration

The process of regeneration involves the formation of a missing structure by the rest of an organism (Wallace, 1981). Urodele amphibians (newts and salamanders) are the only adult vertebrates which have the ability to regenerate their limbs .Upon amputation in any limb region of many larval and adult urodeles, virtually perfect regeneration will occur (Wallace, 1981). Most regeneration studies in the laboratory use the european crested newt, *Triturus cristatus,* the eastern spotted newt of North America, *Notophthalmus viridescens*, or the axolotl, *Ambystoma mexicanum.*

Following amputation of an amphibian limb, the basal cells at the cut edge of the epidermis begin migrating to cover the cut surface. Within a few hours, the plane of amputation becomes covered by an epithelial sheet. The latter increases in thickness, forming a specialized wound epithelium. Cell division is almost nonexistent at this stage, and continued distal migration without local mitosis leads to thickening of the new apical epidermis. The new epidermal covering protects the mesodermal cells which have been exposed by amputation. Local mesoderm cells are damaged by the amputation, and degenerative changes occur there during the next few days. Some mesodermal cells remain viable at the end of this process, and are sometimes said to be "activated" (Wallace,

1981). A limited morphological dedifferentiation of these cells leads to the formation of a blastema (Stocum, 1968). These cells have a generalized mesenchymatous appearance, preventing the former or future identification of their tissue type (Wallace, 1981). However, an anatomically and functionally complete limb will result as these cells redifferentiate missing limb parts in continuity with the stump tissues (Stocum, 1968).

Redifferentiation of the blastema cells occurs in a proximal-to-distal sequence (Stocum, 1984). Cell proliferation is controlled by the wound epithelium and nerve supply (Wallace, 1981). The former is also thought to function in maintaining a proximal-distal gradient of differentiation in the underlying mesenchyme, as well as in controlling the direction of outgrowth (Wallace, 1981). Although it was once proposed that epidermal cells were converted to blastemal mesenchyme, experiments by Chalkley (1954) and Manner (1953) proved that this was not the case.

The wound epithelium appears to be essential for the regeneration process to occur. If a freshly amputated stump is prevented from forming a wound epithelium, blastemal formation will not take place (Wallace, 1981). There are also indications that the growing blastema acts to maintain the apical epidermis. Thus, there is a mutual support between the two structures during the limb regeneration process.

The mechanisms underlying the regenerating ability of urodeles and the loss of this capacity in other vertebrates is not understood. This ability is lost in anuran amphibians by a process also not understood (reviewed by Brockes, 1990). It has

been established that an adequate nerve supply is required for regeneration in adult urodeles and larval anurans. Many researchers believe that an insufficient number or density of nerve fibres in adult anurans and higher vertebrates may be at least partly responsible for the lack of regenerative capacity (reviewed by Wallace, 1981). In the case of adult anurans, the lack of presence of specific gene products may contribute to an explanation of this phenomeneon (Savard *et al.,* 1988). One such candidate gene product is NvHbox1, a homeobox containing gene. NvHbox1 is the newt homologue of the human homeobox gene HHO.c8, and of the *Xenopus* homeobox gene XIHbox1. The transcript of this gene is expressed in the limbs of adult newt but not of adult *Xenopus*. Further study of NvHbox1 may help in elucidating some of the mechanisms involved in regeneration of limbs in adult urodeles (Savard *et al.,* 1988).

Comparison of limb regeneration to limb development

Limb regeneration and the original development of the limb are similar in that they both involve the elaboration of a new pattern of structures (Muneoka and Bryant, 1982). Morphogenesis of a regenerating limb proceeds in a similar manner to that of development of a limb. Thus, the regenerating limb, which is significantly larger in size than the developing limb, is often used as a model for the study of pattern formation. The early blastema has been shown to be similar to the early bud in many respects (reviewed by Bryant and Gardiner, 1992). For example, there are only minor differences in the major proteins synthesized by

mesenchymal cells of the developing bud and the regenerating blastema (Slack, 1982).

In experiments performed to understand the mechanism of pattern formation, these two systems behave similarly. Grafts between regenerating homologous limbs or between regenerating fore- and hindlimbs both lead to the formation of supernumerary limbs. This was also found to be the case when similar grafts were performed on developing leg and wing buds of birds (Muneoka and Bryant, 1982). In order to observe the result of a graft between a regenerating and developing limb, the axolotl was used. The forelimb of this amphibian develops at an accelerated rate relative to that of the hindlimb (Shreckenberg and Jacobson, 1975). Thus, it is possible to perform grafts on axolotls of similar age where the forelimb is regenerating and the hindlimb is developing. A graft of forelimb blastema to hindlimb bud stump led to the formation of supernumerary structures in 62% of grafts. This result supports the idea that the mechanisms controlling pattern formation during limb development are the same as those for limb regeneration (Muneoka and Bryant, 1982).

In contrast, other studies have shown that there may be differences in these two systems. Results from experiments to test the effect of retinol pamitate on developing limbs and regenerating forelimb blastemas suggests that morphogenesis is differentially affected by this compound in these two systems (Scadding and Maden, 1986). RA causes skeletal deletions in intact limb buds, but the effect in amputated limb buds is to "proximalize" positional information. Thus, structures proximal to the plane of amputation

as well as the missing parts will regenerate when an amputated limb is treated with RA (reviewed by Stocum, 1991).

In addition, the origin of the cells in these two systems as well as the role of the nervous system in growth control appear to differ (Fekete and Brockes, 1987). The regenerating blastema has a requirement for nerves that is not present in developing limbs (Singer, 1978). In addition, the regenerating blastema expresses at least four antigens that are not expressed in the embryonic limb bud. These include the wound epithelium-specific antigen WE3 (Tassava *et al*, 1986) as well as the mesenchyme-specific antigen 22/18 (Kintner and Brockes, 1985), and the cytokeratin pair, K8 and K18 (Ferretti *et al,* 1989). Interesting information has been provided by the use of a monoclonal antibody to 22/18 (Ferretti and Brockes, 1987). This antibody recognizes an antigen present on, or associated with intermediate filaments in blastemal cells during the early stages of regeneration. Expression declines after the second week post-amputation, and is no longer detectable with antibody a few weeks before limb regeneration is complete (Kintner and Brockes, 1985). In contrast, less than 1% of mesenchymal cells of the developing limb become stained with this antibody during development (Ferretti and Brockes, 1988). These experiments were performed on newts at similar stages as those of the axolotl used by Muneoka and Bryant (1982) in their grafting experiment (Ferretti and Brockes, 1988). Thus, although the grafting experiments indicate that there are similarities between regenerating and developing limbs, there are several different properties between the two systems. However, the differences that do exist between limb

development and limb regeneration all seem to involve aspects of cell biology that are not directly related to the control of growth and pattern formation. Since all the essential features concerning growth and pattern formation are alike in regenerating and developing limbs (Bryant and Gardiner, 1992), the regenerating limb is often used as a model for the study of pattern formation in the vertebrate limb.

Blastemal morphogenesis

Of central importance in the study of limb regeneration is the determination of how regional patterns distal to the amputation plane are organized from the undifferentiated blastema (reviewed by Stocum, 1984). This involves the elucidation of factors controlling morphogenesis and differentiation of new limb parts from the undifferentiated blastema mesenchyme (Stocum, 1968).

Evidence suggests that the regenerating amphibian limb may contain myogenic precursor cells which are of distinct origin from connective tissue-cartilage precursor cells. In the absence of a regenerative source of muscle precursors, a normal skeletal and tendon pattern still forms (reviewed by Tabin, 1991). The blastema appears to originate from the dedifferentiation of cells in the vicinity of the amputation plane. It was once believed that blastemal cells remained determined to revert to their original tissue specificity when conditions in the regenerating limb allow them to do so (Wallace, 1981). However, it is now believed that all mesodermal tissues are able to contribute cells to the blastema. In a

recent study, two tissue-specific hypomethylation sites in the newt cardioskeletal myosin heavy chain gene have been used as lineage markers to show that cells from both muscle and connective tissue contribute to cartilage formation in regenerating limbs (Casimir et al, 1988). An alternative explanation is that specialized reserve cells contribute to regenerating tissue in the newt (Cameron *et al.,* 1986).

The blastema appears to be an independently differentiating system. It inherits morphogenetic information from parent limb cells (Stocum, 1984).It appears that the identity of the blastema is determined by its location of origin (reviewed by Stocum, 1984). If a forelimb blastema of an urodele is transplanted to an irradiated tail stump, a forelimb will regenerate. Both hindlimb and tail blastemas also regenerate their characteristic structures when transplanted to an ectopic location.

Nerve deflection studies have been used to map the four regeneration territories known to exist on the dorsal surface of the urodele amphibian In these experiments, a major peripheral nerve trunk is cut, and the proximal branch is inserted into the skin in an ectopic location on the dorsal body surface. The injury associated with nerve insertion leads to the formation of a blastema. The type of limb which forms depends on the location of nerve insertion and not on the nerve itself (Guyenot *et al.*, 1948; Kiortsis, 1953, in Wallace, 1981). Thus, it would appear that the limb and tail fields which are established in the embryo persist in the adult urodele (Wallace, 1981). However, a study on the effect of RA on regenerating tails of *Bufo* tadpoles provides evidence that these

fields can be altered during development (Mohanty-Hejmadi *et al*, 1992).

It has been shown that the morphogenetic field for tail formation has the capacity to organize limb formation (Mohanty-Hejmadi *et al*, 1992). Amputation through the middle of the tail of *Bufo andersonii* at the hind-limb bud stage was followed by exposure to a solution of 10 IU per ml of vitamin A palmitate for various lengths of time ranging from 24 hrs. to 144 hrs. In most cases, ectopic supernumerary limb formation from amputated tail stumps was observed. These results suggest that the tail-bud stage of development consists of a broad morphogenetic field which subsequently becomes localized later in development (Mohanty-Hejmadi *et al*, 1992).

It is possible to demonstrate positional specification along the P-D axis of the urodele limb. If a newt limb is amputated at the wrist, a hand will regenerate. If a newt limb is amputated at the shoulder, an entire limb will be reconstructed (Stocum, 1984). This positional memory specifies the proximal boundary of the regenerate such that blastemal cells do not form structures proximal to their point of origin.

Within the limb field, the specification of the developing structure can therefore best be analyzed in terms of its axes. Most studies are concerned with axial specification along the P-D axis of the urodele and along the A-P axis of the chick limb bud (reviewed by Brockes, 1989). The models used to describe how cells aquire information about their position in these two systems are described below.

Models for positional identity

1. Polar coordinate model

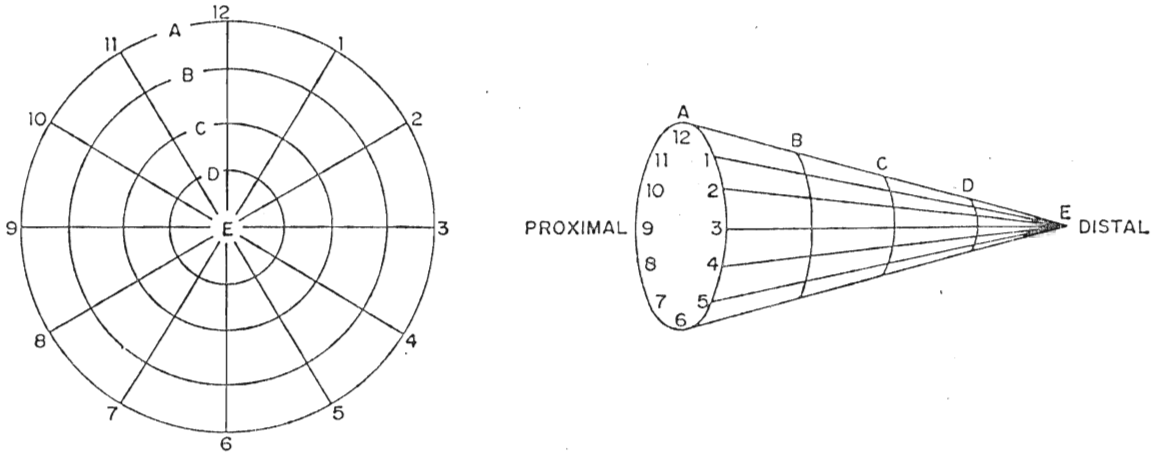
One of the basic problems in developmental biology is how cells are able to acquire and interpret information about their position (Brockes, 1990). There are presently a few models for explaining pattern formation in the vertebrate limb. The polar coordinate model is one means of explaining how patterning occurs (French *et al*, 1976; Bryant *et al.,* 1981). This model is based primarily on studies of regenerating amphibian limbs (Figure 1). Positional information is thought to be specified along polar coordinates in two dimensions, each cell having a positional value with an angular and radial component. In the limb, the radial sequence of positional values corresponds to the P-D axis of the appendage, with the distal tip at the centre of the field. The circumferential sequence of positional values corresponds to the circumferential postional on a cross-section at each P-D level. The most important point to consider in the model is the idea that tissues have the general property of intercalation. Thus, when cells from two normally non-adjacent positions come into contact as a result of grafting or wound healing, the discontinuity in positional value at this site stimulates local growth by cell division. During this growth those positional value which normally lie between the confronted positions are generated. The amount of new growth depends on how much positional disparity exists. Intercalation of circumferential

Figure 1. The polar coordinate model

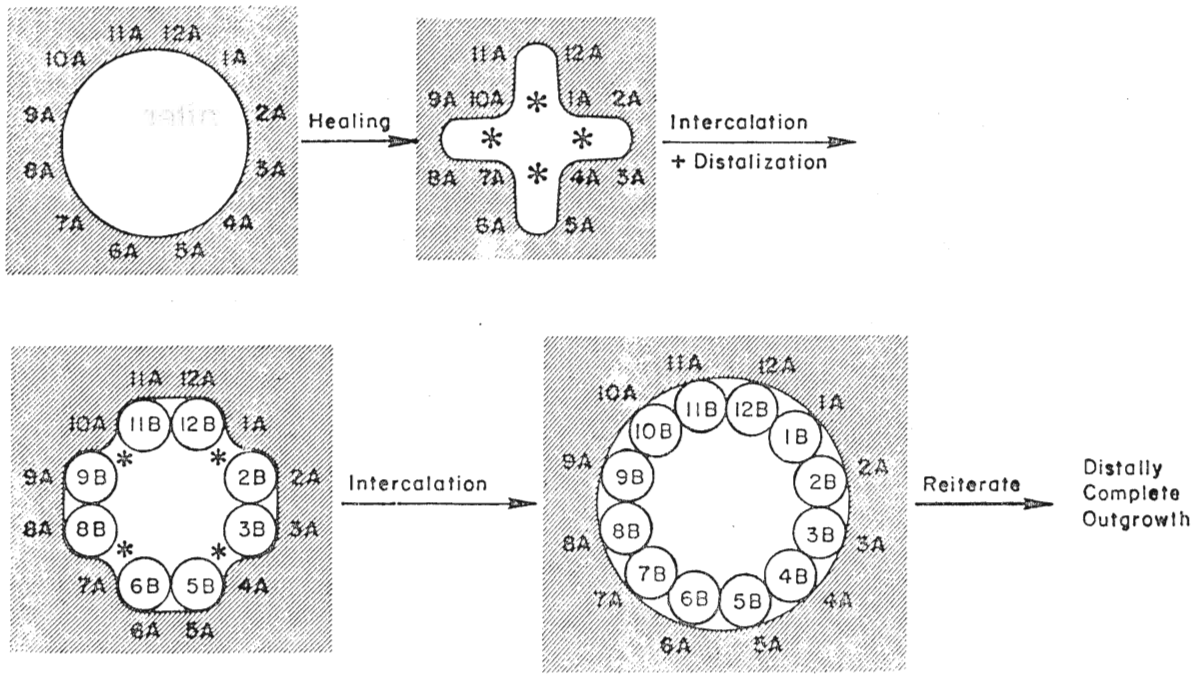
a) Polar coordinates of positional information in a limb. Cells are assumed to have information with respect to their postions along the proximal-distal (A-E) axis, and on a circumference (1-12).

b) Distal outgrowth from a limb circumference, after amputation of levels B, C, D and E. During wound healing, it is proposed that cells from different parts of the circumference come into contact. Circumferential intercalation by the shortest route produces cells with positional values identical to those of the pre-existing adjacent cells, hence the new cells are forced to a more distal level, B in this example (distalization rule). Subsequent intercalation completes level B and reiteration of the whole process generates the remaining distal levels (Bryant and Muneoka, 1986).

a


12
11
A
1
B
10
C
2
D
9
E
3
8
4
7
5
6
A
B
C
D
E
PROXIMAL
DISTAL
12
11
1
10
2
9
3
8
4
7
5
6

b


11A 12A
10A 1A
9A 2A
8A 3A
7A 4A
6A 5A
Healing
Intercalation
+ Distalization
11B 12B
9B 2B
8B 3B
7B 4B
6B 5B
Intercalation
Reiterate
Distally
Complete
Outgrowth

position is thought to occur via the shortest of two possible routes.

Distal outgrowth is thought to occur as cells with differing circumferential positions come together at the tip of the limb. Circumferential intercalation results in a newly generated complete circle of positional values, with the newly generated cells adopting a positional value more distal than that of the pre-exisiting cells. The process continues until an outgrowth that is complete both distally and circumferentially results.

2. Diffusable morphogen model

A second model for explaining how cells acquire positional information is the diffusable morphogen model, originally proposed by Wolpert (1969). Embryology often involves situations where a uniform sheet of similar cells develops into a series of structures. An organizing centre is thought to control position and polarity of these structures. The cells in a field would acquire positional information by reading gradients of diffusable molecules, termed morphogens. Positional information is defined as that information which a cell possesses about its location in the whole organism or organ (Bryant and Muneoka, 1986). This information would allow proper growth and diffferention of tissue (Wolpert, 1969). Cells would respond to varying concentrations of the morphogen and take in different pathways of differentiation, thus producing the characteristic anatomy of the organ concerned . This concept originates with the discovery of the zone of polarizing activity or ZPA in the chick embryo limb bud by Saunders (1968). The ZPA was

thought to act by releasing a diffusable morphogen. One such putative morphogen is retinoic acid (RA) (reviewed by Brockes, 1991). A graded distribution of this molecule across the limb bud would allow specification of the A-P axis of the limb.

Retinoic acid as a possible morphogen

It is well known that RA and its immediate retinoid precursors have the ability to respecify positional identity along an axis in both development and regeneration (reviewed by Brockes, 1989). The effect is both graded and dose-dependent, meaning that positional value is altered continuously within a certain concentration range (Niazi and Saxena, 1978 and Maden, 1982). When forelimb blastemas from *Bufo* tadpoles are exposed to increasing concentrations of retinol palmitate for increasing lengths of time, more and more pattern becomes intercalated until complete limbs form from the distal amputation plane (Maden, 1982). However, due to the fact that RA cannot be synthesized *de novo* by animal cells, and because it has many other biological effects, skepticism arose regarding RA as an endogneous morphogenetic factor (Slack, 1987).

A bead containing RA that is implanted into the anterior of a chick limb bud is able to generate a stable gradient across the limb bud, and is able to cause digit duplications in chick limbs. In extreme cases, the normal digit pattern 2-3-4 is replaced by 4-3-2-2-3-4. The concentration of endogneous RA required to get digit duplications is 20-30nM (Tickle *et al.*, 1985, Eichele and Thaller, 1987). This information led investigators to classify RA as an

endogenous morphogen. It was later shown that the chick limb bud contains RA, that it occurs in a graded distribution from posterior (50nM) to anterior (20nM), and that RA levels correspond to induction thresholds which are known to be present in response to exogenous application of RA (Thaller and Eichele, 1987). This information led to great interest in the possibility that RA and other retinoid metabolites might be used as endogenous morphogens.

Another form of biologically active endogenous retinoid, 3,4-didehydroretinoic acid, has also been discovered (Thaller and Eichele, 1990). This compound is present in amounts six-fold higher than retinoic acid in the chick limb bud. They have similar biological effects and similar distributions *in vivo.* The significance of the existence of two biologically active retinoids in the limb bud remains to be elucidated. Recent reports suggest a slightly different interpretation for the role of RA in alteration of axial specification (reveiwed by Brockes, 1991b). After implantation of a RA-soaked bead into the anterior margin of the chick limb bud, it is possible to remove a wedge of tissue adjacent to the bead and demonstrate polarizing activity after grafting it to a recipient limb bud (Wanek *et al* 1991). Control experiments have ruled out the possibility that the effect is due to a carryover of RA in the grafted tissue (Wanek et al, 1991). Thus, RA may induce the formation of a polarizing region and may not act as a polarizer itself. This study has been complemented by a second one involving a retinoic acid receptor b (Noji *et al*, 1991). After implanting an RA-soaked bead into the anterior margin of a chick limb bud, local RARb induction can be detected by *in situ* hybridization (Noji et al, 1991). Similar

results were observed upon implantation of retinoid beads into the ZPA region (Noji *et al*, 1991). However, no such response was evident when a graft of ZPA to the anterior of the chick limb bud took place (Noji *et al*, 1991). Thus, it appears that RA is not released by the ZPA when either in its native position or after grafting (Brockes, 1991b).

Endongenous retinoic acid in the regenerating limb

In regenerating newt limbs, duplications in limb pattern are possible along the PD axis, as well as the two transverse axes (Thoms and Stocum, 1984). In regenerating urodele limbs, positional respecification has been demonstrated along the P-D axis (reviewed by Brockes, 1990). As mentioned previously, retinoids are able to "proximalize" blastema positional information in this system (Maden, 1982). RA appears to reset the positional memory, causing the blastema to interpret information as if it were present in a more proximal location (Maden, 1982).

A newt anterior half-blastema can only regenerate half limbs, making only the anterior digits. Similarly, a posterior half-blastema only gives rise to posterior structures. Retinoic acid treatment allows an anterior half limb to regenerate a full limb pattern, restoring posterior potential. However, it does not restore anterior potential to a posterior half limb (Kim and Stocum, 1986). RA thus acts to simultaneously posteriorize and proximalize the blastema cells of anterior half limbs and ventralize and proximalize the blastema cells of dorsal half limbs. However, RA inhibits the

regeneration of posterior and ventral half limbs, resulting in the formation of scar tissue (reviewed by Stocum, 1991). This is similar to the chick system where RA transforms anterior mesenchyme to posterior. Also, RA can alter the dorsal/ventral axis during regeneration in an analogous manner to the effects in the A-P axis, allowing dorsal half-blastemas to regenerate ventral as well as dorsal structures (Stocum and Crawford, 1987).

It is not yet known what concentration of RA impinges on blastema cells, or whether this concentration is position-dependent (Brockes, 1990b). It is possible that a source of RA or retinoid precursors may be the epidermis or its associated pigment cells (Baranowitz, 1989), and that this leads to formation of blastema cells (Brockes 1990b). An alternative explanation is that RA may be synthesized from retinol by blastemal mesenchyme cells, as is the case in the chick limb bud polarizing region (Thaller and Eichele, 1988).

Many researchers are not yet convinced that RA or didehydroRA are endogenous morphogens, this being especially true for the urodele blastema (reveiwed by Brockes, 1990). It must yet be proven that there are continous variations in retinoid levels or in cellular responsiveness (Maden, 1988). It must also be shown that axial positions of cells are determined by such variations (Maden, 1988). Regardless of its role as an endogenous morphogen, RA is proving to be an important tool for dissecting the events of pattern formation. Although it has long been recognized that RA is able to affect gene expression, these studies received a major stimulus from the discovery that RA interacts with nuclear receptors of the

steroid/thyroid hormone superfamily (Petkovitch *et al*, 1987; Giguere *et al*, 1987).

Mechanism of action of Retinoic Acid

It is important to determine how RA acts at the molecular level in order to elucidate the mechanisms with which it is able to respecify positional information (Brockes, 1990). The presently accepted mode of action is that RA enters the nucleus and binds to specific nuclear retinoic acid receptors (RARs) (Summerbell and Maden, 1990). The latter are members of the steroid and thyroid hormone receptor superfamily. The receptor-ligand complex then binds to specific DNA sequences on target genes, the RA response element or RARE. This leads to an increase or decrease in transcription (Summerbell and Maden, 1990).

The DNA-binding domain of the steroid and thyroid hormone receptors is highly conserved (Giguere *et al* ,1987). This led investigators to believe that related ligand-inducible transcription factors may be discovered (Giguere, 1987). DNA sequences encoding these domains have been used as probes for related, novel ligand receptors (Giguere *et al*, 1987). This method was used to isolate and characterize the full-length cDNA encoding a receptor for RA (Giguere *et al*, 1987), as well as to screen for hormone nuclear receptors activated by RA in regenerating tissues of the newt (Ragsdale *et al*, 1989). Two functional newt RARs were identified, RARα and RARδ. The former is the newt homologue of the human α receptor, the latter is closely related to the mammalian RARγ

(Ragsdale *et al*, 1989).

As mentioned previously, RA treatment of regenerating limbs can have multiple patterning effects (Ragsdale *et al*,, 1989). These include PD and AP axial respecification, teratogenic-like deletions, and complete inhibition of regeneration. This suggests that at least three different RARs are likely to be present in limb blastemas (Ragsdale,*et al*, 1989).

A second class of compounds that is able to interact with RA and retinoids is the cytoplasmic binding proteins called cellular retinoid (CRBP) or retinoic acid (CRABP) binding proteins (McCormick *et al*, 1988). Two of the four members bind RA: CRABPI and CRABPII (Tabin, 1991). The other two specifically bind retinoids: CRBPI and CRBPII (Tabin, 1991). It is not known how these cytoplasmic receptors function. However, it appears that CRABP occurs in mammalian and chick limb buds in a distribution pattern that is antiparallel to that of RA (Balling, 1991). The highest concentration of CRABP occurs at the anterior limb bud margin of the chick, a location where RA concentration is lowest (Balling, 1991). This situation may help to steepen the gradient of RA in the limb. CRABP may also act as a shuttle, bringing RA to the nucleus where binding to RARs may occur (Summerbell and Maden, 1990)

Recently, another class of receptor that is responsive to RA, the retinoid X receptor (RXR), was isolated. It is substantially different in primary structure and ligand specificity to the RARs (Mangelsdorf *et al*, 1990). The ligand for the three known retinoid X receptors (RXR α, β, γ) is 9-cis RA, although it is activated by but does not bind all-trans RA (Mangelsdorf *et al*, 1992).

It has been shown that the CRBPII gene is a potent target for RXRα. The retinoid X response element found in the promoter of this gene is strongly up-regulated by RXR but not RAR (Mangelsdorf *et al*, 1991). It appears that the RXRs and their cognate ligand may comprise a new regulatory system that plays an important role in vertebrate development.

Although many factors involved in the pathways through which RA acts in the cell have been identified, it is still of interest to determine which molecules are responsible for mediating the morphogenetic effects of RA.

Retinoic acid and gene expression

Although significant advances have been made in understanding the events that occur prior to gene expression in retinoid-induced proximalization of regenerating limbs, very little is known about the events taking place after gene expression (Johnson and Scadding, 1992).

It has been suggested that glycoproteins within blastema cell membranes are responsible for specifying positional values in regenerating limbs (Slack, 1980; Maden, 1983). The axolotl limb regeneration blastema is known to possess a gradient of cell adhesivity in vitro, with the highest adhesivity in the distal region of the blastema (Nardi and Stocum, 1983). RA is able to proximalize these position related differences in cell adhesivity (Crawford and Stocum, 1988).

A study by Johnson and Scadding (1992) has shown that

tunicamycin, a known inhibitor of N-glycosylation of proteins, is able to block the proximalizing effect of RA. Thus, it appears that N-linked glycoproteins play an important role in the specification of pattern in the regenerating limb.

Candidate genes under retinoid control

It would be of great interest to identify genes that are affected by binding of RARs to their transcriptional domains. The products of these genes may play a role in specifying pattern in the limb (Brockes, 1989).

The homeobox-containing genes are members of one class of genes whose expression pattern is altered by exposure to RA. These genes contain a highly conserved 180 bp sequence called the homeobox. This sequence contains a helix-turn-helix DNA binding domain Studies on the effect of RA on the vertebrate Hox4 gene complex have provided interesting results. This gene cluster is expressed in the posteriormost regions of the developing chick and mouse embryos, including the hindlimb bud (Dolle *et al*, 1989). Transcripts are also present in the forelimb bud. The 3'-most gene (Hox4.4) is expressed first at the posterior side of the limb bud. Hox4.4 expression subsequently spreads anteriorly as the bud grows Each successive gene is then sequentially expressed in a more posterior domain, forming a nested set. During growth of the limb bud, expression patterns shift to divide the bud into PD domains (Dolle *et al*, 1989). The effect of RA on this gene complex is to

stimulate the proper temporal sequence in an ectopic location, suggesting that endogenous RA may function to regulate Hox genes (Izpisua-Belmonte *et al*, 1991).

Study of limb development in chicks containing the talpid (ta^3) mutation have provided further insight into the role of homeobox containing genes in setting up anteroposterior asymmetry (Izpisua-Belmonte *et al*, 1992). Chicks which are homozygous for the talpid mutation lack AP polarity. This effect has been correlated with the abnormal extension into anterior areas of the expression domains of those Hox-4 genes that are normally expressed only in posterior mesenchyme. Thus, there is an absence of discrete domains in which cells express different combinations of Hox-4 genes. (Izpisua-Belmonte *et al*, 1992).

As mentioned previously, the expression patterns of some recently isolated homeobox-containing genes in the newt indicate their possible involvement in positional specification along the PD axis (Savard *et al.,* 1988; Tabin, 1989; Brown and Brockes, 1991). However, in contrast to their mammalian and chick homologs, proximalizing doses of RA do not affect transcription of these genes (Savard *et al.,* 1988).

The only newt gene which is currently known to be affected in such a manner is the type II keratin gene, NVKII (Ferretti and Brockes, 1991). NVKII is expressed at higher levels in a distal than in proximal blastemas. This is also the case in normal limbs (Ferretti and Brockes, 1991). A decrease in NVKII mRNA levels in distal blastemas occur upon treatment with RA. The finding that NVKII is present in both the blastema mesenchyme and wound

epithelium, as well as the fact that RA regulates its expression suggests that NVKII positive cells may be involved in specifying positional information (Ferretti and Brockes, 1991).

Carlone *et al* (1992a) have recently identified at least 11 proteins that are potentially involved in specification of pattern in the newt limb. They injected groups of animals with either RA (10μg ml^{-1} , all-trans) in dimethylsulfoxide (DMSO) or with DMSO alone 5 days after bilateral amputations through the wrist (distal group), or mid-humerus (proximal group). The stump or blastema tissue was harvested either 24 hours or 13 days post-injection and subjected to 2-D gel electrophoresis. Proteins whose synthesis was assymetrically distributed along the P-D axis and whose rate of synthesis was altered to proximal levels by proximalizing doses of RA were considered to be good candidate gene products for specifying positional information.

Using this method, it has been possible to identify a number of proteins which are differentially synthesized in proximal versus distal blastemas at two regeneration stages. The rate of synthesis and accumulation of six of these proteins are regulated to "proximal values" in distal regenerates by RA. These proteins may represent members of a cascade that is regulated by RA, and that acts to express positional information in the newt limb regenerate (Carlone *et al*, 1992a). In order to define the role of these six proteins, microsequence information is being obtained, with the goal of cloning the genes encoding these proteins. One candidate protein has an axial distribution and regulation that is opposite to that of the other proteins in the early dedifferentiation and mid bud stages. The

rate of synthesis and accumulation of this protein in limb tissues is increased significantly after a one hour heat shock at 35^{0}C relative to that in control animals similarly treated at ambient temperature. The molecular weight of this protein is 73kD. This evidence suggests that this gene product may be a novel hsp unrelated to the 70kD group of heat shock proteins (Carlone *et al*, 1992b).

Identifying the role of genes involved in pattern formation

Once genes that are potentially involved in pattern formation have been identified, it would be useful to determine more precisely their roles in this process. This may be achieved by expressing their translation products abnormally during limb regeneration. Abnormal expression refers to ectopic expression in cells that would normally not express the gene, gross overexpression in cells that normally do express the gene, or repression of gene expression using an anti-sense RNA message or a blocking antibody (Holt *et al.,* 1990). As stated earlier, it is thought that the mechanisms underlying regeneration and development are similar in nature. Also, the ultimate morphology attained by the limb is the same in both regeneration and embryogenesis. The regenerating blastema is an order of magnitude larger than the developing limb bud, and it is technically easier to isolate tissue from different regions of the limb. Thus, the blastema is an appropriate system for studying pattern formation.

The blastema offers an additional advantage for studying this process. It is possible to remove blastema cells from the

regenerating limb and transfer them to tissue culture (Ferretti and Brockes, 1988), or to remove the entire blastema from the limb perform various manipulations on the cells and return them to an irradiated limb stump. In order to study the regenerative process in a more controlled environment, blastema cells are often grown in organ culture (Jabaily *et al.,* 1982). However, there are some inherent problems with using newt limb tissue culture cells for this purpose. The culture of adult urodele cells, especially on a long-term basis, has always presented numerous problems, including difficulties with dissociation, establishment of adequate culture conditions, and frequent contaminations (Ferretti and Brockes, 1988). Cell culture is also a very expensive technique to use. In addition, blastema cells divide every 54 hours, and may become polyploid after long-term culture. Blastema cells in tissue culture may also lose positional cues after being dissociated for an extended period of time. Thus, it appears to be advantageous to perform transfection on an intact blastema which has been removed from its limb, and return it to an irradiated limb as soon as possible. Irradiation is necessary to minimize any cellular contribution from limb stump tissue to the patterning effects contributed by transgenic cells.

An even more advantageous situation would be to misexpress genes of interest in an intact blastema *in vivo*, that is, to manipulate cells of a blastema which remains attached to the limb. This system would be relatively easy to work with, and it would also be possible to avoid possible contaminations that are frequent with *in vitro* manipulations.

Gene Targeting

Gene targeting in newt limb cells is much more difficult than in other systems. For example, in order to perform gene targeting in mice, it is possible to isolate embryo-derived stem (ES) cells from mouse blastocysts (Capecchi, 1989). Once the ES cells are determined to be pluripotent, DNA of interest can be introduced into them. The ES cells can then be microinjected into the blastocoel cavity of an embryo (Capecchi, 1989). The blastocyst can then be surgically transfered into the uterus of a pseudopregnant mouse, and development allowed to progress to birth (Capecchi, 1989). Such a technique may be used to identify genes involved in establishing positional information in mouse development (Capecchi, 1989). Unfortunately, it has not been possible to isolate ES cells from urodele amphibians. Thus other techniques must be explored for introducing genes of interest into these animals.

Transfection

Transfection involves the delivery of functional polynucleotides into living cells (Felgner and Ringold, 1989). A gene of interest is inserted into a modified bacterial plasmid such as pUC with the proper regulatory sequences (e.g. promoters, enhancers). Various methods have been developed for introducing plasmid DNA into cells of interest, although all methods do not work well in all cell types.

Methods for introducing DNA into mammalian cells

One of the earliest methods used to transfect mammalian cells is DEAE-dextran (Howard *et al.,* 1971). Initially, investigators used this method to aid in the introduction of SV40 DNA into 3T3 nonpermissive mouse cells. DEAE-dextran interacts with DNA directly, and may adhere to the cell surface. DNA then enters the cells by pinocytosis. The polycations can be toxic to the cells, however, (Howard *et al.,* 1971) and this does not appear to be a very reproducible technique (Graham and van der Eb, 1973).

Another technique for mammalian cell transfection is that of calcium phosphate precipitation. This technique was developed based on the principle that divalent cations are essential for bacterial cell transformation. These investigators determined that sufficiently high concentrations of calcium ions appeared to enhance uptake of Adenovirus Type 5 DNA by human KB cells. A plaque assay was used to show that calcium phosphate precipitation is 100-fold more efficient than DEAE-dextran. However, the technique is very sensitive (Graham and van der Eb, 1973) and does not result in the stable transfection of cells when used with newt limb blastema cells in culture (Tabin, personal communication).

Electroporation is often the method of choice for DNA transfection with higher eukaryotic cells *in vitro.* It is known that cells in suspension or culture become transiently permeablized upon exposure to some critical electrical field strength and duration (Tekle *et al.,* 1991). Although this technique has proven superior to

more conventional DNA transfection methods for many cell lines (Tekle *et al.,* 1991), blastema cells do not readily survive electroporation attempts.

Presently, microinjection of blastema cells in culture is the most efficient method for transfection of blastema cells in culture. It has many disadvantages however. The technique is tedious, time consuming and expensive. Also, microinjection can only be used with cells in a monolayer. Since the blastema must be dissociated, there is a possibility that the cells will lose their positional cues. It has recently been reported that the use of a particle gun to introduce gold-coated DNA into blastema cell cultures has met with some success (Brockes, personal communication). In addition, this technique could be modified to introduce the DNA into blastema cells *in situ.* A major drawback however, is the high cost of this particular approach.

Lipofection

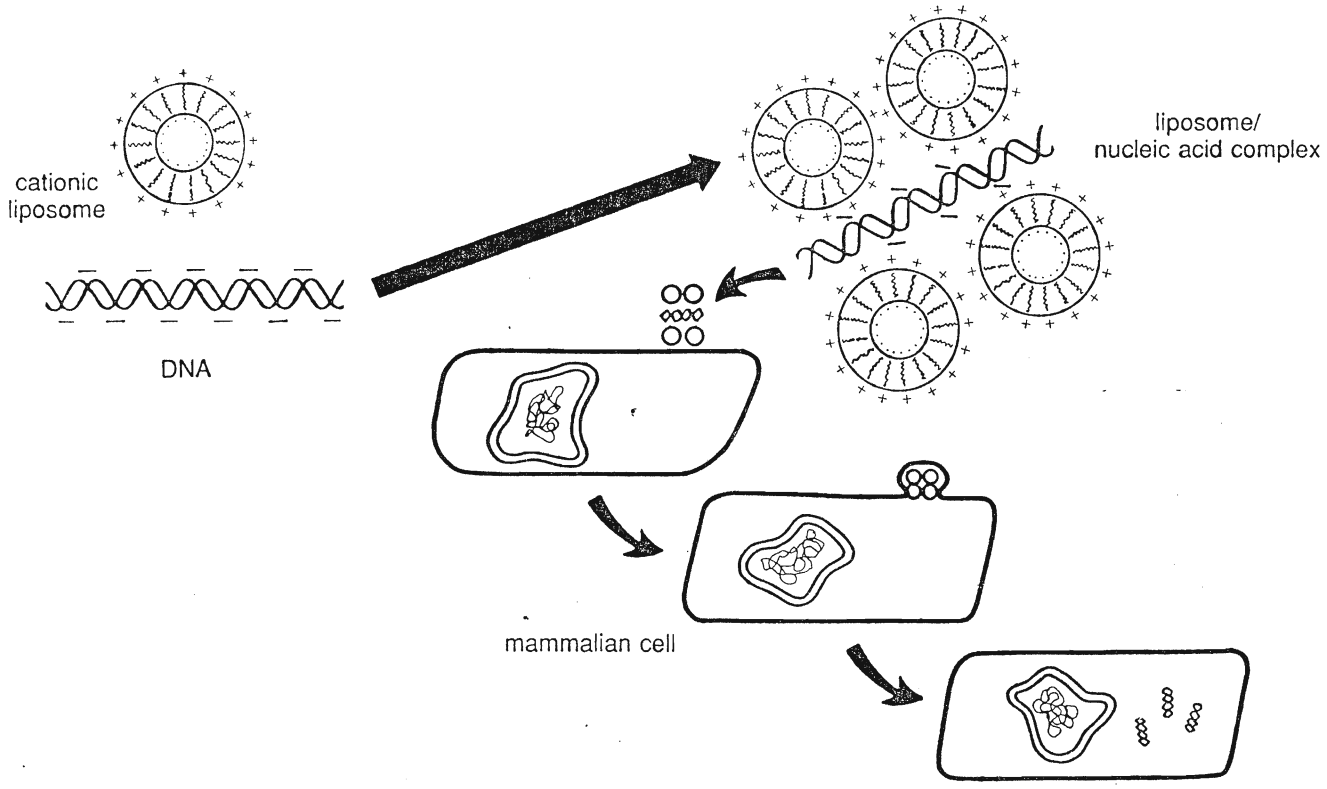
Another method with which successful DNA transfection of blastema cells in culture has been successful is lipofection (Tabin, personal communication). Lipofection is a relatively new DNA transfection technique (Felgner *et al.,* 1987). It is simple, highly reproducible and efficient and easier to use than other techniques commonly used today. Since it is difficult to find cationic, bilayer forming lipids that form stable liposomes, a synthetic lipid DOTMA (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride) has been designed and synthesized. This lipid, either alone or in

combination with neutral phospholipids, spontaneously forms multilamellar liposomes that can be sonicated to form small, unilamellar vesicles. When DNA is mixed with these liposomes, ionic interactions between negatively charged DNA and positively charged DOTMA leads to complex formation (Figure 2). These vesicles are appropriate tools for transfection since they can act as cationic mediators of the transfection process via spontaneous complex formation with DNA and the cell surface. Fusion with the plasma membrane allows efficient delivery of functionally active polynucleotides into the cell cytoplasm (Chang, 1988).

In contrast to other transfection procedures, lipofection is a relatively mild delivery system (Holt, *et al.,* 1990). For some cell lines, transfection efficiencies 5 to 100-fold higher than that observed with either calcium phosphate or DEAE-dextran have been reported (Holt *et al.,* 1990).

Lipofection has been successfully used to introduce DNA into *Xenopus* embryos. A plasmid (pRSVL) containing a cDNA encoding the luciferase gene of the firefly *Photinus pyralis* has been targeted to presumptive eye and brain neuroepithelium using this method. A significant fraction of embryonic neurons became transfected after exposure to DNA and lipofectin, with the estimated transfection efficiency being 1% (Holt, *et al.,* 1990). Using immunocytochemistry, it was found that transfected neural cells showed varying levels of luciferase expression. Cells expressing luciferase at low levels appeared to have luciferase expression localized to peroxisomes. Cells expressing high levels of luciferase displayed diffuse and often extremely bright labelling throughout

Figure 2. Illustration of liposome-mediated transfection (Felgner *et al*, 1987).


cationic
liposome
DNA
liposome/
nucleic acid complex
mammalian cell

the cell bodies and along dendrites and axons (Holt, *et al.,* 1990). The latter group of cells constituted approximately 40% of the total transfected population. The investigators calculated that roughly 0.14pg of luciferase protein was present per transfected cell (Holt, *et al.,* 1990). Transfection rates increased significantly when tissues were treated with a mild enzyme solution to remove epithelial tissue prior to lipofection. The skin epithelium appears to provide a barrier to the transfection process. The external mucousal layer may prevent liposomes from interacting and fusing with plasma membranes (Holt, *et al.,* 1990).

Monitoring luciferase expression during transfection showed that activity rose steeply, to peak by around 24-48 hr. At 56 hrs., activity had usually declined to about half the maximal value. Since the experiments were performed on isolated heads which usually do not survive beyond 4-5 days, the drop in activity after 56 hrs. may represent a decline in cell viability. Therefore, long-term experiments were performed on intact animals.

Whole animals that were transfected with pRSVL were monitored for luciferase activity over a 14 day period. In these experiments, luciferase activity rose during the first 4 days, remained at its peak value from 4 to 8 days, then dropped to just less than half the peak value by 14 days. Another type of experiment designed to test for long-term expression of luciferase involved injecting a DNA-Lipofectin mixture directly into the lumen of embryonic brains. Luciferase activity rose to peak levels by 2 days, remained high until 8 days, and still exhibited significant activity at 28 days. This suggests that some of the DNA may have become

stably integrated into the host genome of some cells (Holt *et al*, 1990).

For some applications, it would be useful to be able to inject DNA directly into tissues *in vivo* (Wolff *et al*, 1990). This has been accomplished using various methods including formulations of DNA encapsulated in liposomes (Nicolau *et al*, 1983) and calcium phophate coprecipitated DNA (Benvenisty and Reshef 1986). It has been recently shown that it is possible to inject pure DNA or RNA directly into mouse skeletal muscle (Wolff *et al.,* 1990). Mouse quadricep muscle was injected with either 100μg DNA or RNA plasmid, both containing the chloramphenicol acetyl transferase (CAT) reporter gene (Wolff *et al.,* 1990). The resulting enzyme activity was comparable to that achieved by transfecting fibroblasts with the corresponding constructs *in vitro* (Wolff *et al.,* 1990). Knowing this result, it seems promising to attempt direct injection of DNA into newt limb blastemas *in vivo*. One group was able to successfully inject a mixture of DNA-lipofectin into *Xenopus* embryonic brains. Using this method they estimated a transfection efficiency of 0.1%, approximately ten-fold lower than that achieved by incubating isolated brain preparations in lipofectin (Holt, *et al.,* 1990).

Based on the success with lipofection of *Xenopus* tissue and direct injection of cDNA into mouse muscle tissue, we thought it may be useful to use these techniques for transfection of newt blastema mesenchyme.

The goals of my thesis were to:

a) Attempt to improve on the reported transfection efficiency of

lipofection. This involved varying the ratio of DNA to Lipofectin, varying the concentration of DNA, and optimizing the duration of lipofection.

b) To introduce DNA into the mesenchyme cells of a regenerating newt blastema by *in vivo* injection. The DNA was injected with and without prior complex formation with Lipofectin, and a comparison was made between these two methods.

MATERIALS AND METHODS

BACTERIAL CELL CULTURE TECHNIQUES

Bacterial Strain

Escherichia coli strain DH5α (Bethesda Research Laboratories) was used as a host for the plasmids used to transfect blastema cells. Its genotype is: F-,*end*AI, *hsd*R17 (r-,m+), *sup*E44, *thi*-1, λ–, *rec*A1, *gyr*A96, *rel*A1, d(*arg*F-*lac*zya)U169, ϕ80d*lac*zdM15.

Propagation and maintenance of bacterial cultures

All bacteria were grown in sterile LB broth (1% Bactotryptone, Difco; 0.5% Bactoyeast, Difco; 1% Sodium Chloride, BDH) with continous shaking in either a floor shaker (New Brunswick Scientific, Incubator Shaker, Model G25) for large scale cultures, or in a water bath shaker (New Brunswick Scientific, Gyratory Water Bath Shaker, Model G76) for cultures of 50ml of less. In order to obtain a clonal isolate of a bacterial population, overnight liquid cultures were streaked on agar plates containing ampicillin. For short term storage, bacteria were kept at 4°C on agar plates. For long-term storage, 1 ml aliquots containing 15% glycerol (BDH) were kept at -70°C. Viable bacteria were recovered by thawing an aliquot and transferring 10μl to 5ml of LB.

Transformation of bacterial cells

Transformation was carried out essentially as described by Sambrook *et al.* (1989). 1ml of fresh overnight DH5α culture was diluted in 50ml sterile LB medium and grown to log phase (O.D.600 ~1). This was achieved in 2.5-3 hrs at 37°C. The bacterial culture was then centrifuged at 4000 rpm (IEC Centra-7R) for 15 min at 4°C. Cell pellets were resuspended in 20ml $CaCl_2$ solution (100mM $CaCl_2$) and maintained on ice for 1hr or overnight. Bacteria were collected once again by centrifugation as above and the cell pellets resuspended in 1ml of $CaCl_2$ solution.

Approximately 10μg plasmid DNA was added to 100μl of competent DH5α in an eppendorf tube. The tube was gently tapped to mix the contents, and subsequently placed on ice for 10 mins. The contents were then heat-shocked at 42°C for 45 sec, diluted with 500μl of LB, and incubated at 37°C for 45 min. Finally, aliquots of the bacteria were plated onto LB agar plates supplemented with 100μl of ampicillin (25 mg/ml).

PLASMIDS USED FOR TRANSFECTION

The plasmid constructs used for transfection experiments are represented by the maps in fig1. The plasmids pSVluc (gift from C. Tabin, Boston) and pRSVL (de Wet *et al.*, 1987, gift from S. Subramani, San Diego) contain the reporter gene luciferase from the firefly *Photinus pyralis*. The luciferase gene in pSVluc is under the control of the SV40 promoter. In pRSVL, this gene is under the

control of the LTR (long terminal repeat) promoter of the Rous sarcoma virus . The construct phspPTlacZpA (gift from J. Rossant, Toronto) contains the reporter gene β-galactosidase from the bacteria *E. coli.* A mammalian heat shock (hsp68) promoter is present upstream of the reporter gene.

ISOLATION OF PLASMID DNA

Small scale isolation of plasmid DNA

Mini-prep plasmid DNA preparations were carried out essentially as a modified version of that described by Sambrook *et al*, 1989). Colonies were picked from agar plates and used to inoculate 5ml of LB supplemented with ampicillin. These cultures were incubated overnight in a water bath shaker at 37°C. 1.5ml of culture was transfered to a 1.5ml eppendorf tube, the culture was centrifuged for 20 sec at 12,000 rpm, and the bacterial pellet was resuspended in 150µl Tris-EDTA (25mM Tris-Cl, pH8.0; 10mM EDTA). Three hundred microlitres of alkaline SDS were then added to the tube (0.2N NaOH, 1% SDS) to lyse spheroplasts and denature cellular proteins. The proteins were subsequently precipitated with the addition of 225µl of acidic potassium acetate (3M, pH 4.8) and the tube was then mixed gently. The tube was then spun at 12000 rpm for 5 min. at room temperature. In the meantime, 500µl of phenol/chloroform/isoamyl alcohol (24:24:1) was added to a second eppendorf tube. After spinning, 600µl of supernatant was added to the phenol, mixed, and vortexed gently. The tube was then spun for 2

min, and 600µl of the top layer was then transfered to a fresh tube. The tube was filled with cold isopropanol, mixed, and placed at -20°C for 15 min. The tube was then spun for 15 min, and the supernatant discarded. The tube was inverted for 1min to dry the pellet, and the pellet was then washed twice with cold 80% ethanol. The pellet was dryed in the speed-vac for approximately 2 min, and resuspended in 40µl sterile water containing 50µg/µl RNAaseA (Boehringer Mannheim). The tube was incubated in a heating block at 37°C for 15min, then at 70°C for 10min. A 5-8µl aliquot was normally sufficient for restriction enzyme analysis and gel electrophoresis.

Large Scale isolation of Plasmid DNA

For large scale plasmid DNA preparations, a 5ml culture was grown overnight as described above. A 1ml aliquot was then used to inoculate 500ml of LB containing ampicillin, and the culture was again grown overnight. The culture was then transfered to 250ml bottles, and centrifuged at 6000 rpm for 5 min at 4°C. The supernatant was then poured off, and the pellet resuspended in 10ml of lysozyme solution (50mM glucose; 10mM EDTA; 24mM Tris-HCl, pH8) containing 50mg lysozyme, and incubated on ice for 1hr. Subsequently, 20ml of alkaline SDS was added, and the bottles were left at room temperature for 5-10 min. Fifteen ml of acidic potassium acetate was then added, and the bottles were placed on ice once again for 1hr. The bottles were spun at 10,000 rpm for 10 min at 4°C, and the supernatant was filtered through cheesecloth

into 30ml Corex tubes. DNA was precipitated with 95% ethanol, and the tubes were placed at -20^{0}C for 1hr. The tubes were spun at 10,000 rpm for 10 min (870 IEC rotor), the ethanol poured off, and the tubes inverted to allow the pellets to dry. The pellets were then resuspended in 5ml TE buffer.

Cesium chloride purification

The plasmid DNA was then further purified through a cesium chloride gradient with a modified procedure from Sambrook *et al.* (1989). First, the volume of the DNA solution was measured. For every 1ml, 0.96 g solid CsCl was added. This was then mixed gently until the salt was dissolved. A 1 ml aliquot was then weighed to ensure that the final density of the solution was approximately 1.57mg/ml. Subsequently, 35µl of ethidium bromide (10mg/ml) was added to each microcentrifuge tube, giving a final concentration of 10µg/µl. The DNA solution was added to each tube, and the tubes were sealed. The solution was then centrifuged in the Beckman TL-100 ultramicrocentrifuge at 65,000 rpm for 20 hrs at 22^{0}C.

The lower band of DNA (the circular plasmid DNA; Sambrook *et al.*, 1989) was collected by inserting a 18 gauge needle through the tube, and extracting the DNA into a 1ml syringe. The ethidium bromide was removed from the DNA by adding a equal volume of water saturated butanol, shaking the tube, and removing the top layer. This process was repeated until all the pink colour disappeared from the DNA solution. The CsCl was then removed by adding 2 volumes of water, followed by 6 volumes of ethanol to

precipitate the DNA. The tube was then centrifuged, the pellet allowed to dry, and then dissolved in an appropriate volume of TE buffer.

Restriction enzyme analysis

Restriction enzyme analysis was performed to confirm the identity and integrity of plasmid DNA after the isolation and purification steps. Digestions were performed with the approriate restriction enzymes at 37^{0}C for 2 hrs. The reaction mixtures contained 10X Restriction Buffer (Pharmacia), DNA, and 0.1 to 1.0 units of enzyme per μg of DNA, depending on the suppliers recommendations. The total reaction mixture was 10-20μl. The reaction was stopped with the addition of 2-4μl of stop buffer (20% glycerol; 2% SDS; 0.5% Bromophenol blue).

Agarose gel electrophoresis

The digested DNA samples were loaded onto 0.7% agarose gels immersed in Tris-acetate buffer (40mM Tris-acetate, 10mM EDTA, pH 8.0). Electrophoresis proceeded at 70V, allowing adequate separation of the fragments in about 2 hrs. Following electrophoresis, the gel was stained with ethidium bromide, placed on a UV transilluminator, and photographed with a mounted Polaroid Land camera with Polaroid type 57 film.

ANIMALS

Care and Maintenance

Adult red-spotted newts, *Notophthalmus viridescens*, (Charles Sullivan, Nashville, Tennesse) were maintained in dechlorinated tap water and fed twice weekly with live Tubifex sp. Prior to amputation, the animals were anaesthetized in m-aminobenzoic acid that had beed diluted to 1X from a 10X stock (5g per 100ml dechlorinated water). Amputations were performed by cutting at the wrist perpendicular to the long axis of the limb, using a razor blade. The animals were then allowed to recover, and limb regeneration allowed to take place to the mid-bud stage. The limbs were then re-amputated for *in vitro* studies, or left intact for *in vivo* transfection.

TRANSFECTION

Lipofection

1.Blastemas

In order to perform lipofection of blastema mesenchyme *in vitro*, it was necessary to remove the tissue from the limb prior to any manipulations. The animals were anaesthetized, placed in a 0.1% solution of chloramine T for 15 sec., then dipped in 95% ethanol and finally placed in sterile dH_20. The blastema was then removed from

the animal using a razor blade, and placed in Ca^{++}-Mg^{++} free PBS (1.4M NaCl, 30mM KCl, 80mM Na_2HPO_4, 15mM KH_2PO_4) for 20 min . The epithelium was then removed with the aid of a dissecting microscope and forceps.

The DNA transfection procedure used was essentially a modified version of that described by Felgner *et al.* (1987) to transfect cultured cells. The cesium chloride purified plasmid DNA was mixed with Lipofectin. The latter is a synthetic cationic lipid preparation comprising N-[1-(2,3-dioleoxyl)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and dioleoxyl-phosphatidylethanolamine (DOPE) (Bethesda research Laboratories). In a typical experiment, a desired amount of DNA was mixed with 100% Modified Ringers (MR) (0.1% NaCl, .2mM KCl, 2mM $CaCl_2 \cdot 2H_20$, 1mM $MgCl_2 \cdot 6H_20$, 50ug/mL gentamycin, 25U/mL mycostatin (pH to 7.4) to give a volume of 62.5µl in one well of a 96 well Microtest III tissue culture plate. In a separate well, a desired amount of Lipofectin was mixed with 100% MR to give a volume of 62.5µl. The diluted lipofectin solution was then added dropwise to the diluted DNA solution. The solution was mixed 15 min prior to addition of tissue to allow time for DNA/Lipofectin complexes to form. Transfection plates were placed on a rotating table and incubated for 20 hrs. The blastemas (4 per well) were then transfered to 100% MR using forceps, and incubated a further 48 hrs. Assays (described below) were then performed to determine the amount of enzyme activity present in the blastemas.

2.Cultured cells

HeLa cells are a transformed human fibroblast cell line (Scherer *et al*, 1953). Lipofection of HeLa cells followed by an assay for enzyme activity was carried out as a positive control. Monolayer cell cultures were grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum and 1x antibiotic-antimycotic (10µg/mL penicillin G, 0.25µg/mL streptomycin, 0.85% amphotericin). Prior to lipofection, the cells (ideally ~80% confluent) were rinsed twice with serum-free Opti-MEM I Medium to remove serum from the culture. The DNA and lipofectin were mixed separately in Opti-MEM to give a volume of 0.5 mL for each. The DNA and lipofectin solutions were then mixed together and added dropwise to the cells. The monolayer cultures were incubated at 37°C for 5 hrs. Following the transfection, 3mL DMEM supplemented with 10% fetal bovine serum was added to the cells. The cells were then incubated a further 24 hrs. and assayed for enzyme activity.

Direct injections

Animals were again amputated at the wrist, and blastemas allowed to grow to the midbud stage (approximately 2.5 weeks). The animals were anaesthetized prior to injections. Plasmid DNA either alone or complexed with lipofectin was injected in 2 sites in the blastema using a 10µl Hamilton syringe fitted with a 33 gauge needle. The DNA and lipofectin were mixed together in a 1:3 ratio without prior dilution. A small amount of fast-green dye was added

to ensure that the DNA and DNA/Lipofectin was not leaking from the tissue. Each injection contained ~200nl of DNA or ~500nl of DNA/Lipofectin complex. The blastemas were removed from the limb as described previously four days post injection, except in one set of experiments where fourteen days were allowed prior to isolation of blastemas from the limb. Four blastemas were pooled together and assayed for enzyme activity.

ENZYME ASSAYS

Luciferase Assays

1.Blastemas

In order to perform luciferase assays on the blastemas, it was necessary to break open the cells of the tissues first. The tissues were placed in mini-homogenizers containing 100µl of extraction buffer (100mM K_2HPO4, 1mM dithiothreitol). The tissue was then homogenized until the mixture was of uniform consistency. An 18 gauge needle was then used to shear the tissue further, and the homogenate was then removed to an eppendorf tube. The minihomogenizer was then rinsed with an additional 100µl of extraction buffer and added to the eppendorf tube, giving a final volume of ~200µl.

Luciferase assays were then performed according to the method of deWet *et al* (1987). A 50µl or 75µl aliquot of homogenate was added to an eppendorf tube containing 350µl assay buffer (5mM

ATP, 15mM $MgSO_4$, 25mM glycylglycine, pH 7.8) The sample was then added to a glass tube and placed into the sample well of a luminometer (LKB 1250). The reaction was initiated with the addition of 100µl of 10mM luciferin (Sigma) with vigorous mixing. The amount of luciferase present was determined by extrapolating from a standard curve. The latter involved measuring the relative luminescence in millivolts (mV) of samples containing known amounts of purified firefly luciferase (Sigma). The luminometer values were recored in mV integrated over 10 sec. for a period of 300 sec.. The highest value for each time period was used to determine the amount of luciferase present in each sample using the standard curve.

2.Cultured cells

Luciferase assays were performed on HeLa cells according to the method of deWet *et al* (1987). Each 35mm plate of transfected HeLa cells was washed three times in Ca^{++}-Mg^{++} free PBS and the cells harvested in 700µl extraction buffer. The cells were pelleted by centrifugation and resuspended in 100µl extraction buffer. Cells were lysed by three cycles of freezing on dry ice and thawing at 37°C. Cell debris was pelleted by centrifugation in a microcentrifuge for 5min at 4°C, and a 10µl aliqout of supernatant was assayed for luciferase activity as described above.

β-galactosidase assay

1.Blastemas

The plasmid construct used in this case (phspPTlacZpA) contained a heat shock promoter. Therefore, the animals or blastemas were placed in dechlorinated water which had been prewarmed to 34.5°C in the New Brunswick Floor Shaker for 1 hr. They were then placed back at room temperature, where a period of approximately 15 hrs. was allowed for gene expression to occur. After this period, the blastemas were incubated for 15 min. in 100µl of fixative containing 20mg/mL paraformaldehyde in PIPES buffer (0.1M PIPES [piperazine-N,N'-bis-{2-ethane-sulfonic acid}], 2mM $MgCl_2$, 1.25mM EGTA [ethylene glycol-bis {3-aminoethyl ether} N,N,N',N'-tetra-acetic acid). The tissues were then washed 3 times in PBS solution containing 1mM $MgSO_4$ for 5 min. each. The tissues were then placed in a well containing 1mL of staining solution (35mM potassium ferricyanide, 35mM potassium ferrocyanide, 2mM $MgCl_2$, 2.5µl of 1mg/mL X-gal {5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside}(diluted from a 40mg/ml stock solution in dimethyl formamide). The tissue was stained overnight at 37°C in the dark with shaking. The presence of enzyme activity could then be visualized by the appearance of an insoluble blue precipitate.

2.Cultured cells

HeLa cells were fixed and assayed in a similar manner. The culture plates were washed twice with PBS and fixed with paraformaldehyde as above. The cells were washed with PBS again and stained as above.

PHOTOGRAPHY

Blastemas which contained cells that had stained positive for the enzyme β-galactosidase were photographed using a microscope (Leitz, Diaplan) with a camera attachment. The blastemas were placed on a glass slide and a cover slip was used to compress the tissue so that the majority of the blue cells were within the same focal plane. The blastemas were then photographed at either 100X or 400X magnification.

TISSUE SECTIONING

Blastemas which appeared to express significant amounts of β-galactosidase activity were cryopreserved and sectioned. Tissue Tek OCT compound (Miles Scientific) was used to bind the blastema tissue to a metal block that was suspended over liquid nitrogen. The

blastema was then cut into 10μm sections using a cryomicrotome that had been cooled to -20°C.

PROTEIN ASSAYS

Protein assays were performed according to the method of Bradford (1976). After the blastemas were homogenized in extraction buffer, a 5μl aliquot of supernatant was diluted with 75μl dH_20. Subsequently, 20μl of Bio-Rad Dye reagent concentrate was added to the tube and placed at room temperature for 1 hr. The absorbance of the sample at 595nm was measured using a Beckman DU50 spectrophotometer.

The amount of protein present in the sample was determined by extrapolation from a standard curve obtained by measuring the absorbance of samples containing known quantities of bovine serum albumin.

RESULTS

Quantitation of luciferase activity in cells and tissues

Prior to each luciferase assay, it was necessary to generate a standard curve using purified luciferase (Sigma). A typical standard curve is illustrated in Figure 3., and was used to determine the amount of luciferase present in each sample (Table 1 and as described in Materials and Methods).

In order to determine if the luminescence detected from a sample was due to the enzymatic activity of the luciferase, an aliquot of the sample was incubated at 95°C for 5 min. prior to assay. Heat inactivation resulted in background levels of luminescence. Also, when blastema mesenchyme was incubated *in vitro* with DNA alone, no luciferase activity could be detected (Figure 5). This was also the case when blastema mesenchyme was incubated *in vitro* with Lipofectin alone (data not shown).

Cultured cells

Cultured HeLa cells were transfected according to the method of Felgner *et al*, (1987). Using the plasmid pSVluc, it was possible to measure 1-2 pg luciferase per μg total cell protein (Table 2). Using the plasmid phspPTlacZpA, 20-25% of the cells were stained blue, indicating that they expressed the enzyme β-galactosidase (Figure 4).

Figure 3. Standard curve of light emission in mV from samples containing purified luciferase and the appropriate substrates. This graph was used to determine the amount of luciferase present in blastema mesenchyme that had been transfected with plasmid DNA containing a luciferase reporter gene.

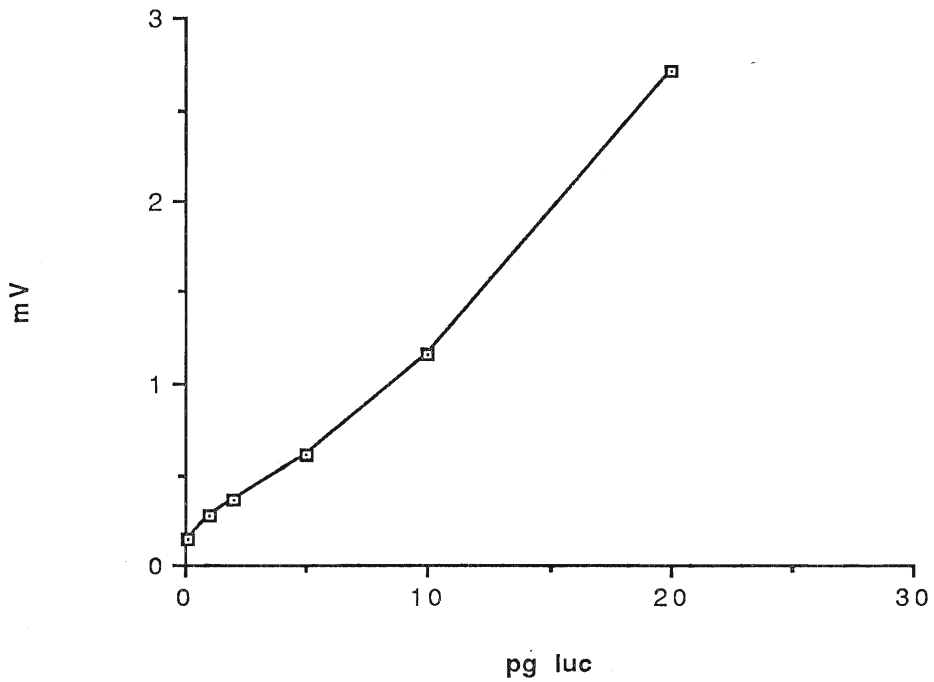
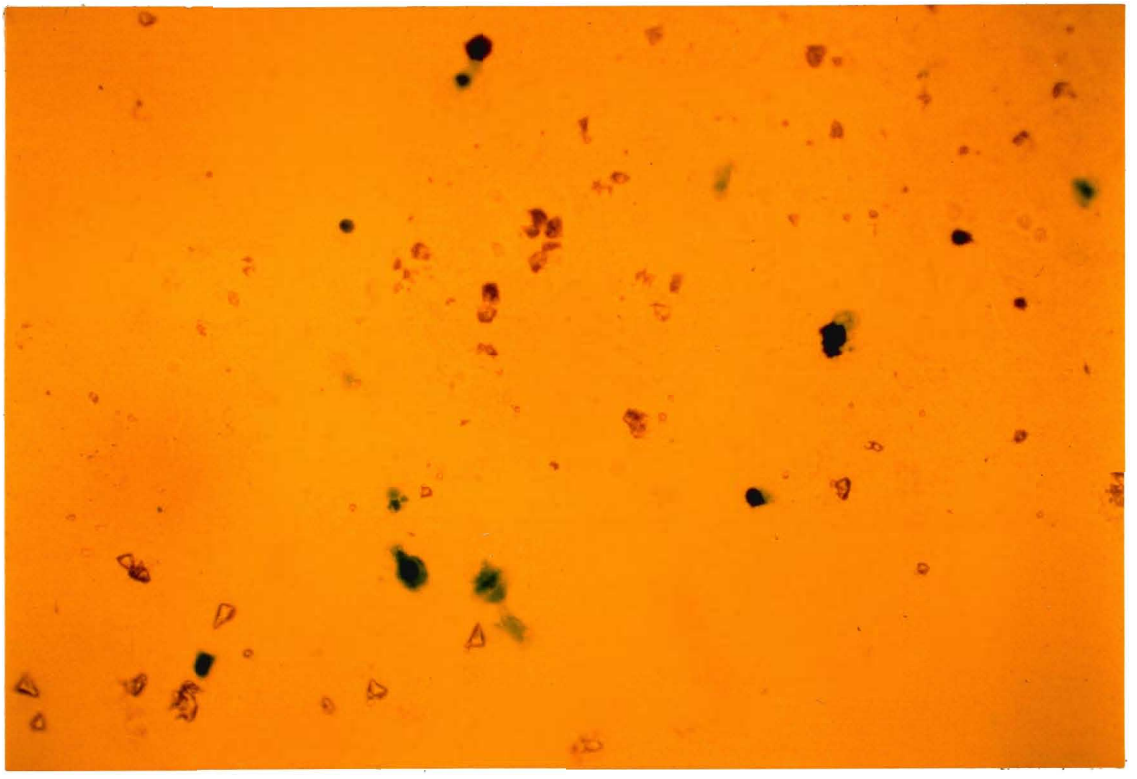

3
2
1
0
mV
0
10
20
30
pg luc

Figure 4. Expression of β-galactosidase in cultured HeLa cells after lipofection with the plasmid phspPTlacZpA in a 1:3 ratio with Lipofectin (w/w) (x300).



In vitro

The ratio of nucleic acid to Lipofectin is known to be critical for optimizing transfection efficiency in cultured cells (Felgner *et al*, 1987). To determine the optimal ratio for newt blastema mesenchyme, the amount of DNA was kept constant (3.75μg), while the amount of lipofectin was varied (Figure 5). As the ratio of DNA to Lipofectin was decreased, the transfection efficiency increased, with maximal luciferase expression occuring with a ratio of 1:3 DNA to Lipofectin. As the ratio of DNA to Lipofectin was decreased further, the amount of luciferase expression also decreased. When the ratio was 1:3.5, the level of expression was approximately two-thirds maximal value; when the ratio was 1:4, the level of expression was approximately one-fiftieth maximal value (Table 3).

In order to test if the total amount of DNA in the transfection medium limits transfection efficiency, the amount of DNA was increased along with the amount of lipofectin so that a 1:3 ratio of DNA to Lipofectin was maintained. Luciferase activity increased with increasing concentrations of DNA (Figure 6). When blastema tissue was incubated with 22.5μg of DNA, it was possible to measure 1.44pg luciferase per μg total blastema protein (Table 4).

Lipofection of blastema mesenchyme was also performed using the plasmid pRSVL. Although the results obtained for both plasmids were quite variable, pSVluc appeared to give consistently better results than pRSVL when equal amounts of plasmid were incubated with lipofectin in a 1:3 ratio (Figure 11 and Table 6).

Figure 5. Expression of luciferase activity in newt blastema mesenchyme after lipofection with varying ratios of DNA:Lipofectin (w/w). The amount of plasmid DNA (pSVluc) was kept constant at 3.75μg, while the amount of Lipofectin was varied in each sample.

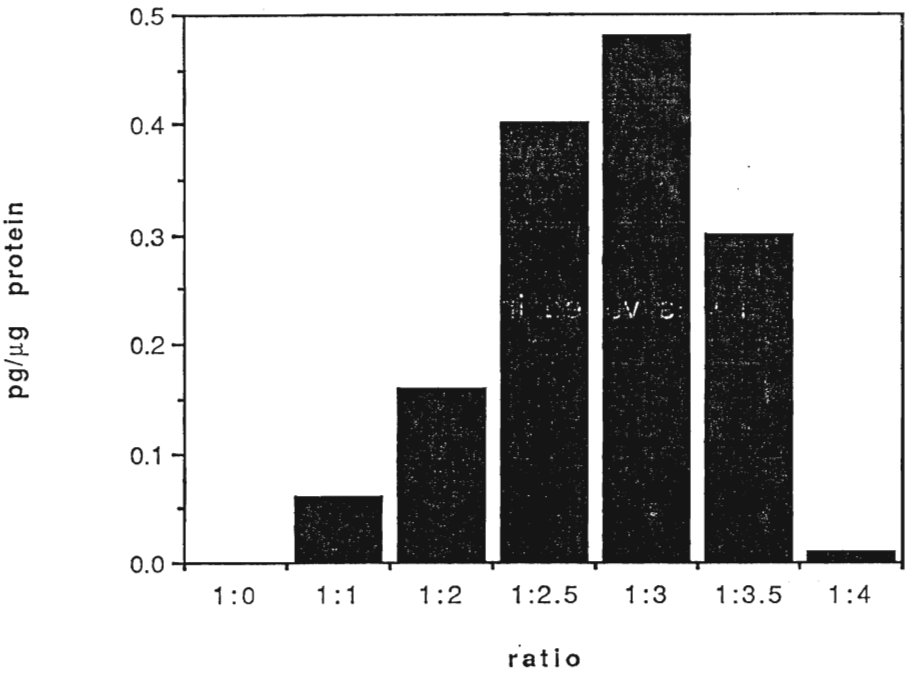

0.5
0.4
0.3
0.2
0.1
0.0
pg/µg protein
1:0
1:1
1:2
1:2.5
1:3
1:3.5
1:4
ratio

Figure 6. Expression of luciferase activity in newt blastema mesenchyme after lipofection using increasing amounts of DNA. The amount Lipofectin was increased also so that a 1:3 ratio of DNA:Lipofectin (w/w) could be maintained.

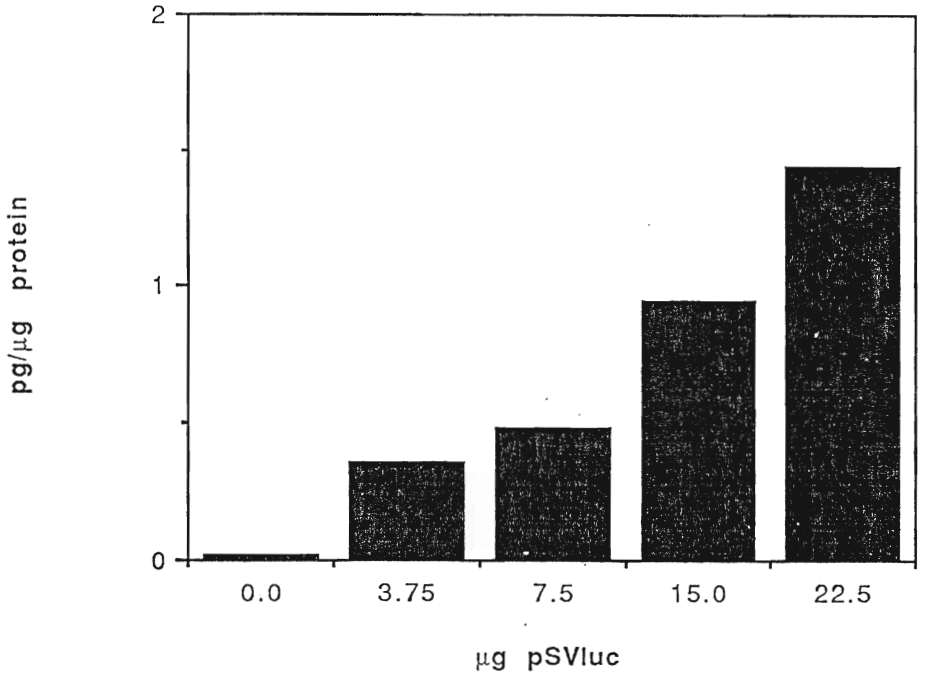
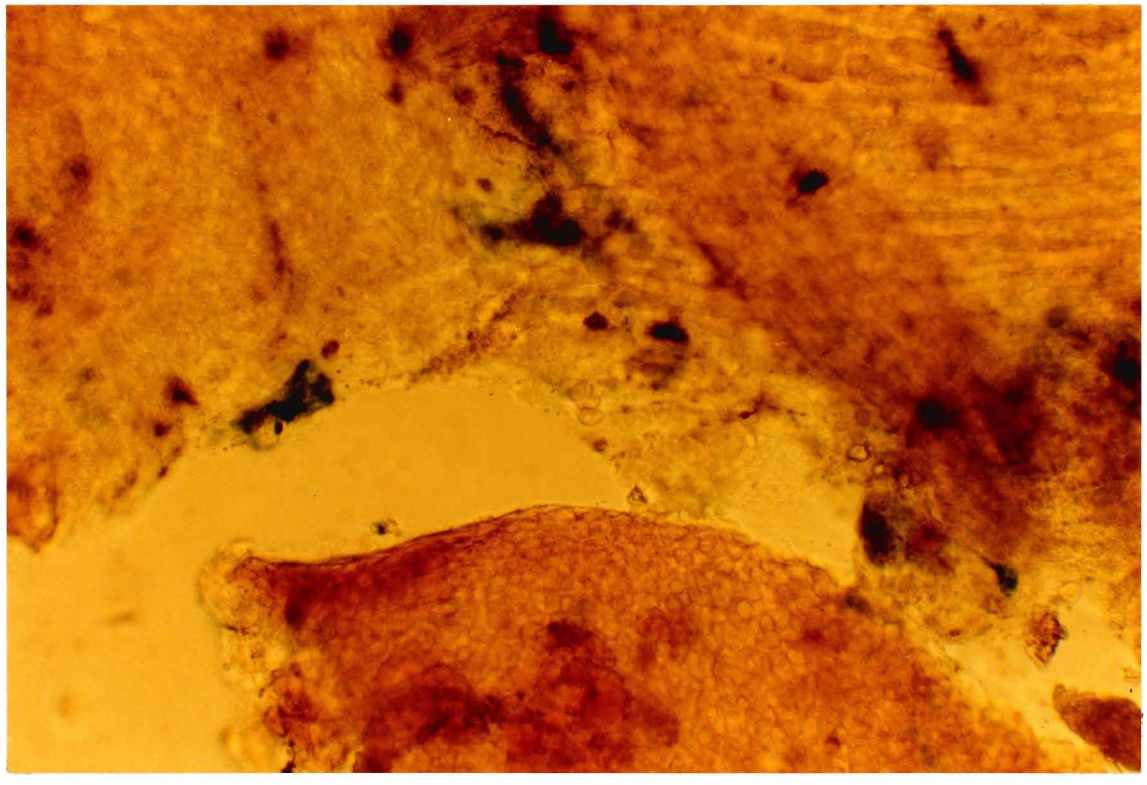

2
1
0
pg/µg protein
0.0
3.75
7.5
15.0
22.5
µg pSVluc

Figure 7. Expression of β-galactosidase after lipofection with phspPTlacZpA in a 1:3 ratio with Lipofectin (w/w). The tissue was heat-shocked 15 hrs. prior to assay for enzyme activity (x300).



The plasmid phspPTlacZpA was also used to transfect blastema mesenchyme *in vitro*. The assay for β-galactosidase activity allows quantitation of the number of cells expressing the transgene as a percentage of total cells in culture or in a section of blastema tissue. When blastema mesenchyme was incubated with phspPTlacZpA and Lipofectin in a 1:3 ratio and heat-shocked 15 hrs. prior to assay for enzyme activity, it was possible to count 10 to 15 blue-stained cells per blastema (Figure 7). In tissue which had not been heat-shocked prior to assay, there were typically 2 or 3 blue-stained cells per blastema (Figure 8). Tissue incubated with either DNA or Lipofectin alone did not express any enzyme activity (Figure 9).

In vivo

The plasmids pSVluc and pRSVL were injected into 2 sites of the distal region of blastema forelimb mesenchyme under the wound epithelium. The plasmids were injected with and without prior complex formation with Lipofectin. Again, these results were variable, but injection of 1.6μg or 5μg of pSVluc resulted in consistently higher transfection efficiency than injection of equal amounts of pRSVL (Figure 10). Injection of 1.6μg of DNA complexed with lipofectin resulted in a slightly higher transfection efficiency than when 1.6μg of DNA was injected alone (Figures 10 and 11). However, the levels of expression are very low in all cases, therefore it is difficult to conclude which method of direct

Figure 8. Expression of β-galactosidase after lipofection with phspPTlacZpA in a 1:3 ratio with Lipofectin (w/w). The tissue was not heat-shocked prior to assay for enzyme activity (x300).

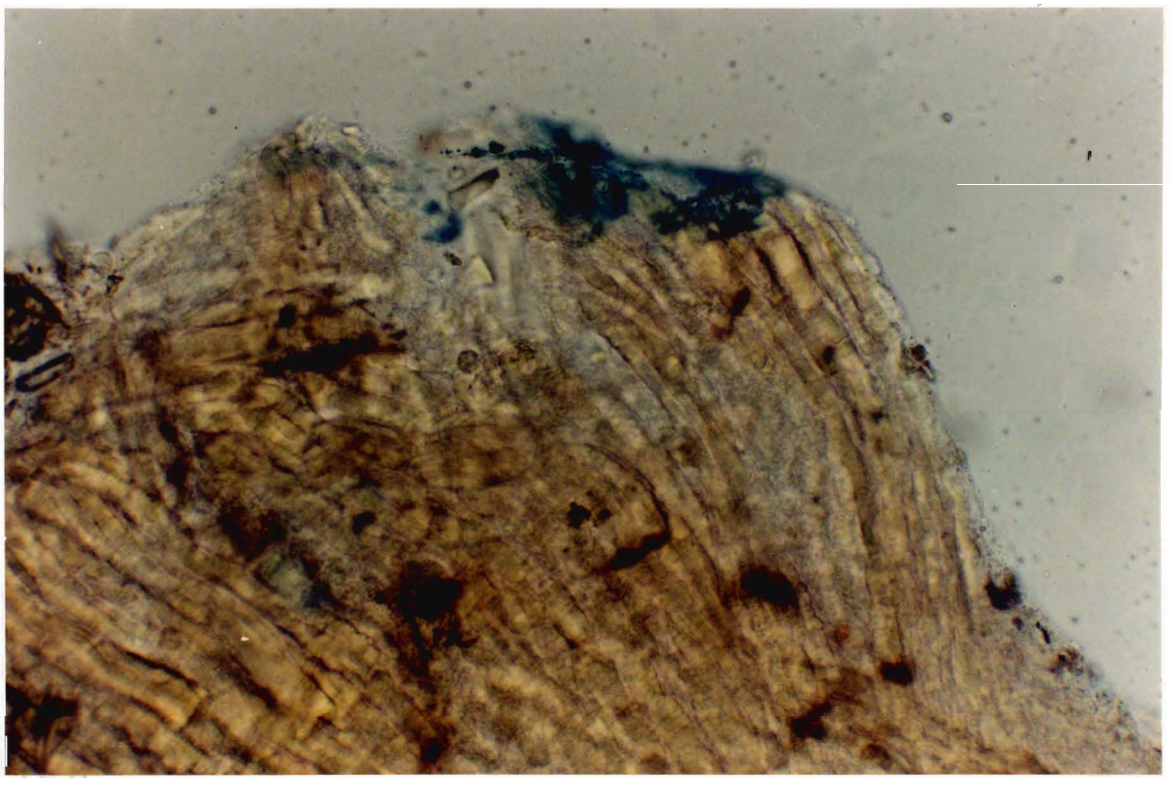


Figure 9. Blastema mesenchyme incubated with the plasmid phspPTlacZpA *in vitro*. It was not possible to detect any blue cells in this case (x300).

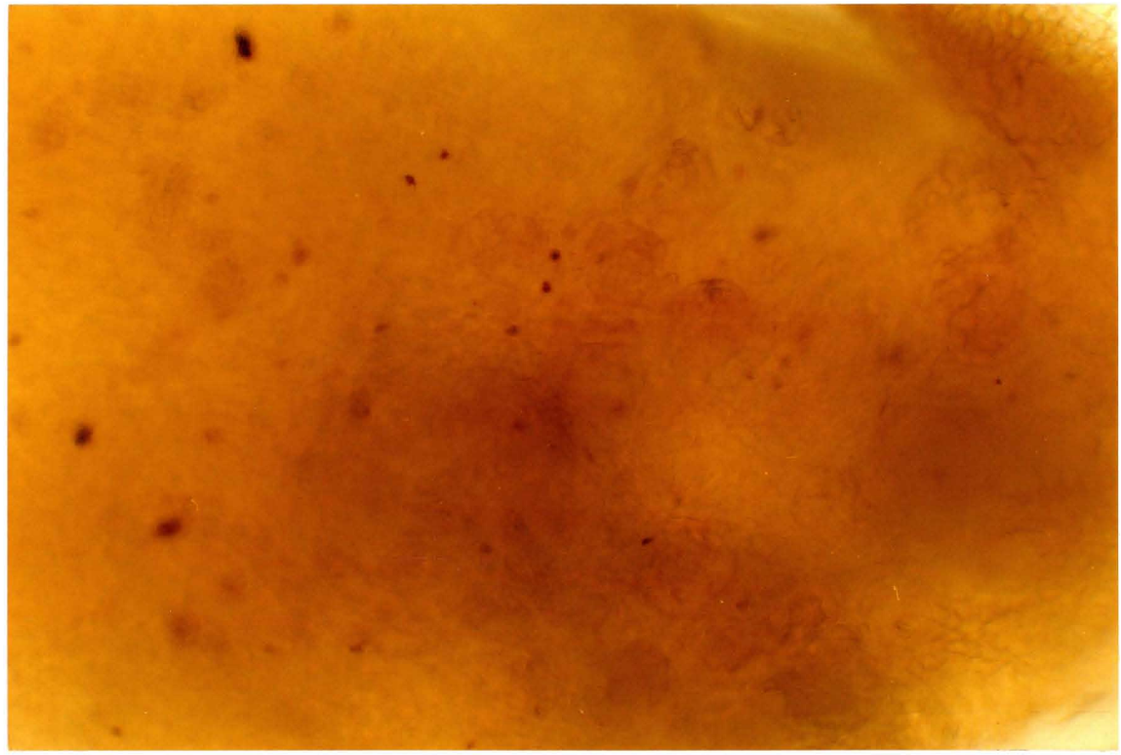


Figure 10. Expression of luciferase activity 4 days after injection of either pSVluc or pRSVL into newt blastema mesenchyme.

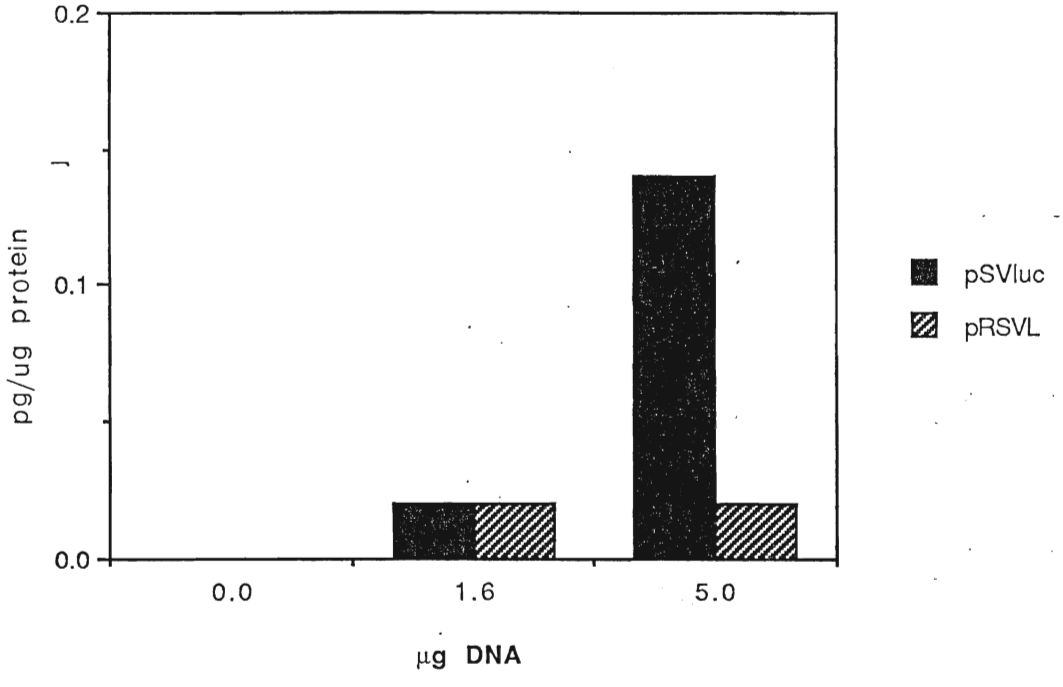
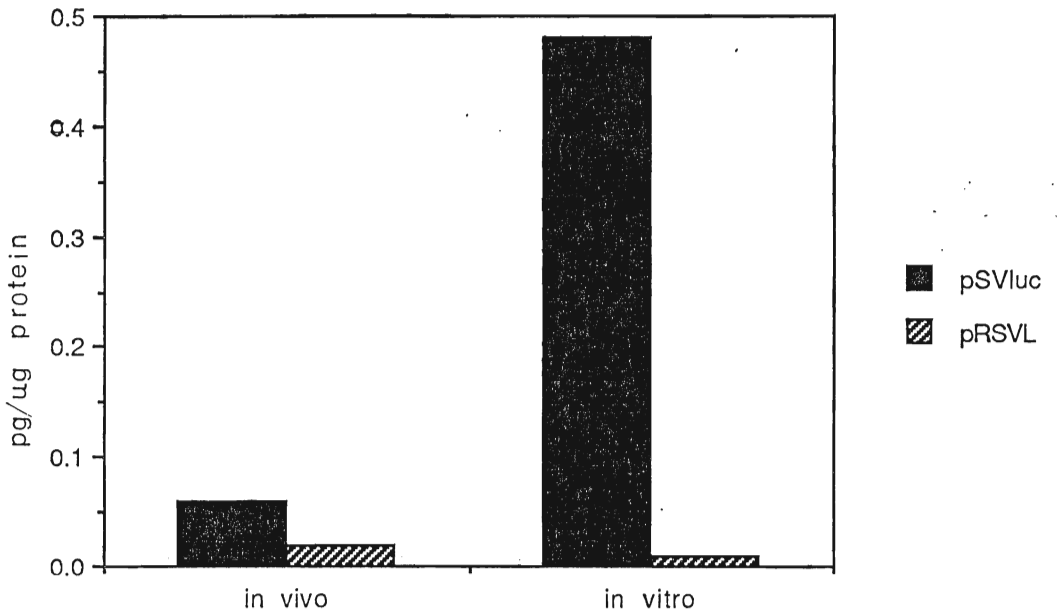

0.2
0.1
0.0
pg/ug protein
0.0
1.6
5.0
µg DNA
pSVluc
pRSVL

Figure 11. Graph showing comparison of luciferase activity obtained from the plasmids pSVluc and pRSVL both *in vivo* and *in vitro*. For the *in vivo* experiment, plasmid was injected into newt blastema mesenchyme in a 1:3 ratio with Lipofectin (w/w). The *in vitro* data is from Figure 6 where blastema tissue was incubated with plasmid DNA in a 1:3 ratio with Lipofectin


0.5
0.4
0.3
0.2
0.1
0.0
pg/ug protein
in vivo
in vitro
pSVluc
pRSVL

injection is better when these plasmids are used. It is significant however, that direct injection of pSVluc or pRSVL alone resulted in measurable albeit low levels of luciferase activity. As mentioned, this is not the case *in vitro* , where incubation of blastema tissue with either plasmid alone did not result in any measurable levels of enzyme activity.

When phspPTlacZpA was incubated with Lipofectin prior to injection into blastema mesenchyme, the results are similar to those obtained *in vitro*. Only a few blastema cells were stained blue per injection site (Figure 12).

In contrast, direct injection of 0.2 to 0.5μg of this plasmid into blastema mesenchyme resulted in significant patchy expression localized to the injection site (Figures 13 and 14). In many cases, more than 50% of all blastema cells in any injection site and 10% of all blastema cells in a 10μm section expressed β-galactosidase activity. The number of blue cells in a tissue section were counted under a light microscope. Enzyme expression in the cells at any site was variable. As seen in Figure 14, some cells are stained dark blue, presumably indicating high levels of expression. Other cells are only lightly stained, and probably correspond to cells expressing low levels of enzyme activity. Again, it is significant that injection of phshPTlacZpA without Lipofectin resulted in significant levels of β-galactosidase activity. This was not the case *in vitro*, where incubation of this plasmid alone with blastema tissue did not result in any detectable enzyme expression.

In order to examine the long-term pattern and stability of β-galactosidase expression in blastema mesenchyme, an assay for

enzyme activity was performed 14 days after injection of 0.5μg of phspPTlacZpA. The expression of β-galactosidase was considerably less than after 4 days, but was still significant in patches associated with the injection site (Figure 15).

Figure 12. Photomicrograph of blastema mesenchyme whole mount from regenerate four days after injection of phspPTlacZpA mixed with lipofectin in a 1:3 ratio. Blue cells represent expression of beta-galactosidase activity after heat shock and histochemical staining.

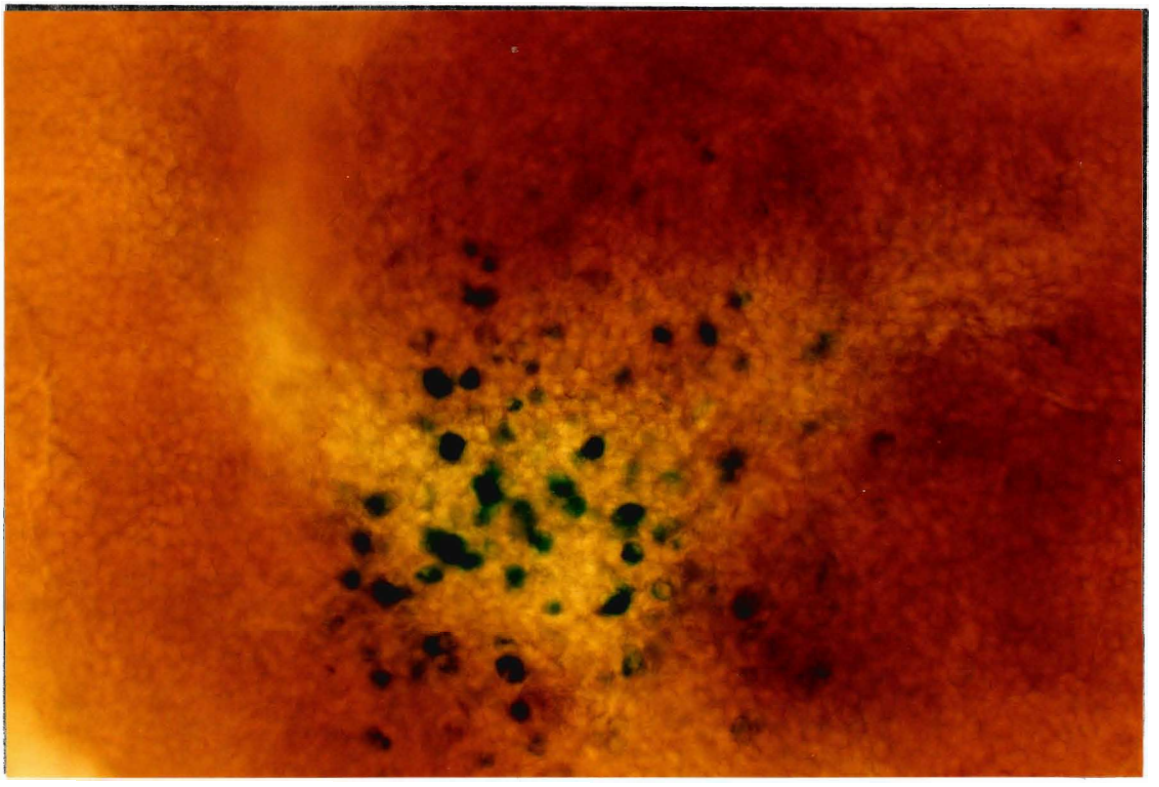


Figure 13. *In situ* cytochemical staining of blastema cells for *E. coli* β-galactosidase activity. The assay for enzyme activity was performed 4 days after injection of 0.5μg of phspPTlacZpA into blastema mesenchyme tissue (x300).

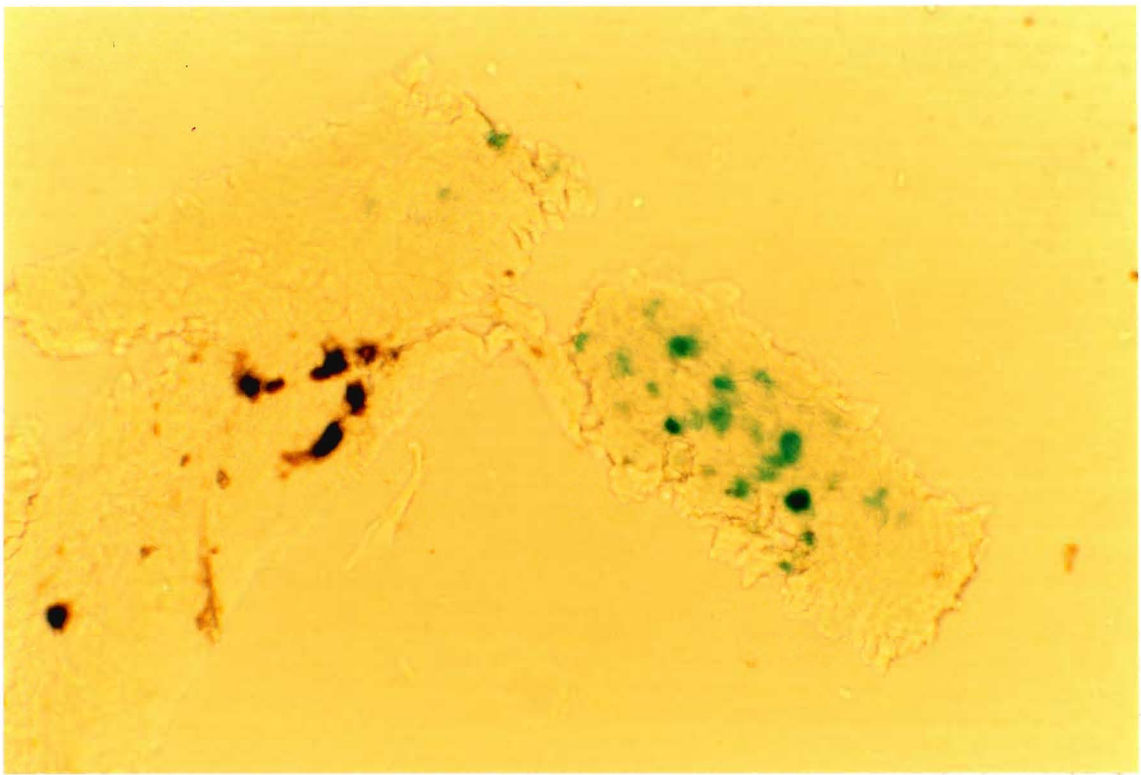


Figure 14. *In situ* cytochemical staining of blastema cells for *E. coli* β-galactosidase activity. The assay for enzyme activity was performed 4 days after injection of 0.5μg of phspPTlacZpA into blastema mesenchyme tissue. The tissue was frozen and cut into 10μm sections (x700).

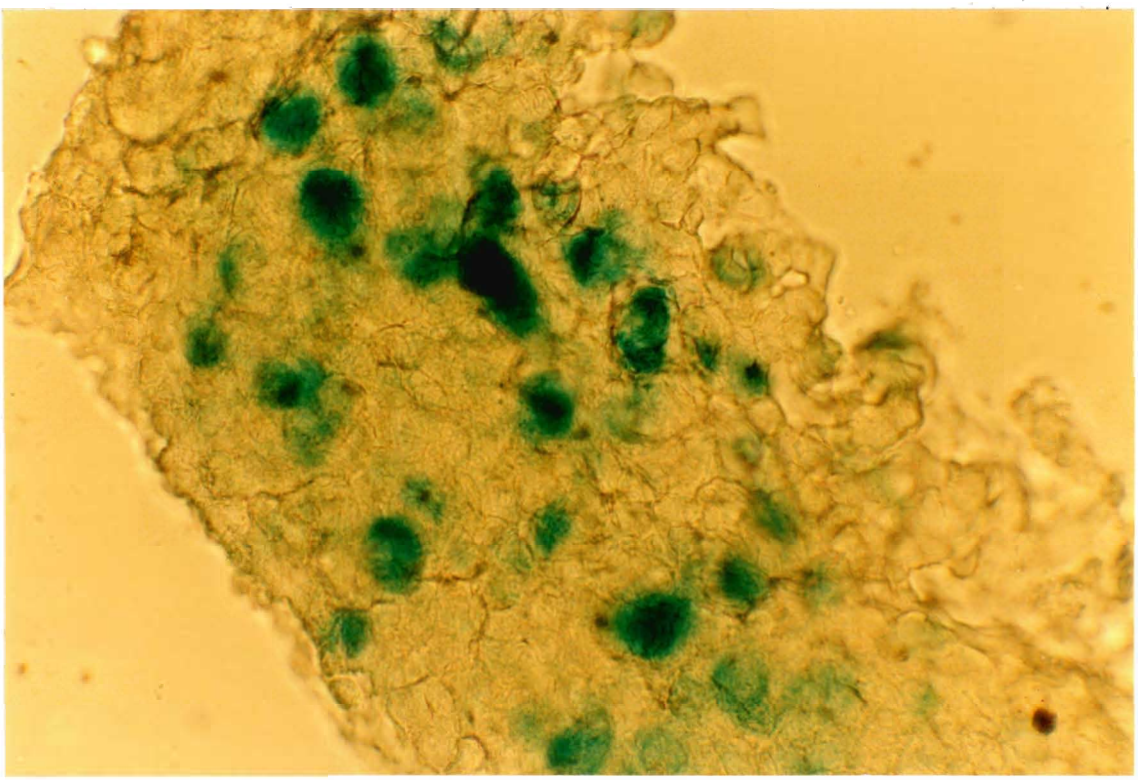
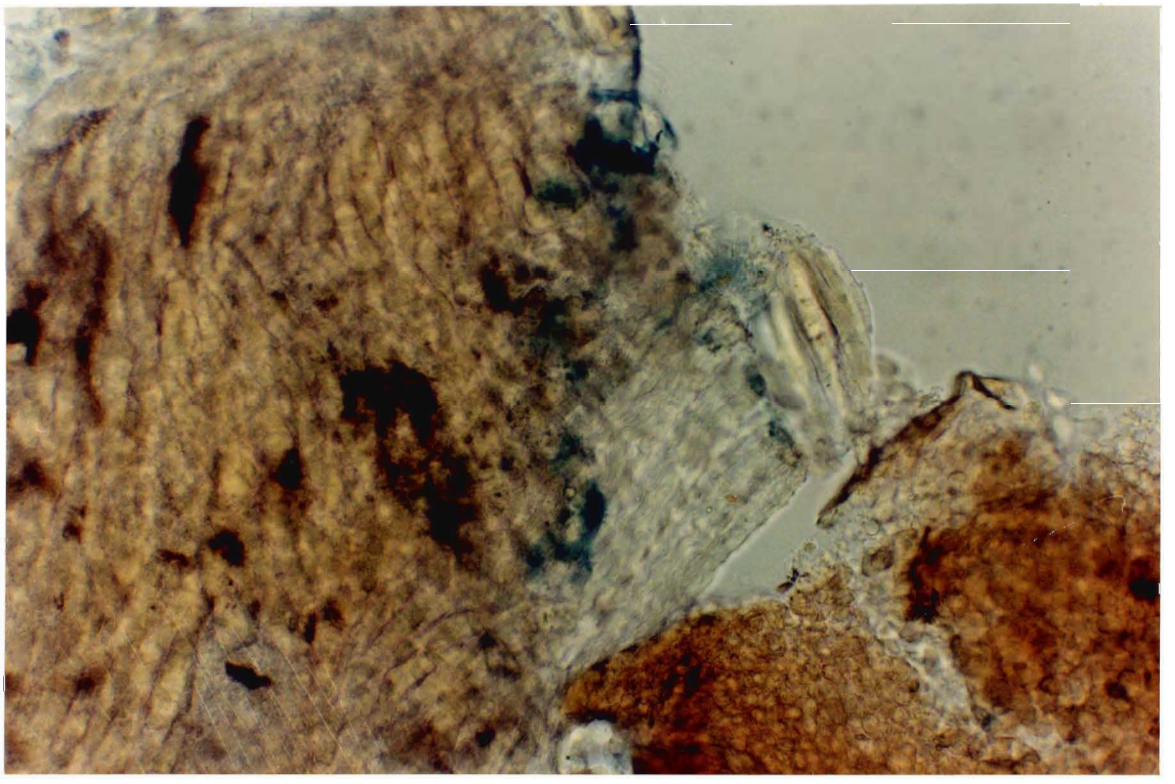


Figure 15. Expression of β-galactosidase activity 14 days after injection of phspPTlacZpA into blastema mesenchyme (x300).



DISCUSSION

The techniques of lipofection and direct gene transfer have been used to introduce plasmid DNA into mid-bud stage blastema mesenchyme. Using the technique of lipofection, the optimal ratio of DNA:Lipofectin was found to be 1:3. This is similar to that obtained by Holt *et al* (1990) using Xenopus embryonic neural tissue. This result is consistent with the view that an excess of DNA reduces the net positive charge of DNA-Lipofectin complexes, and inhibits their interaction with the negatively charged cell membrane (Felgner and Ringold, 1989).

In order to test the effect of increasing DNA concentration on transfection efficiency, the amount of DNA added per culture was increased up to 22.5μg. The amount of DNA was not increased any further since large volumes of Lipofectin would be required. The latter is very expensive and was in limited supply. In the Xenopus CNS, luciferase activity increased with increasing amounts of DNA and peaked at 70μg DNA per 210μg Lipofectin (Holt *et al*, 1990). It would be interesting to determine if blastema mesenchyme tissue would require a similar amount of DNA for optimal transfection efficiency *in vitro*.

The amount of luciferase activity expressed was variable in all experiments. There are a number of factors which may be responsible for this variability, including differential susceptibility of individual blastema mesenchyme to lipofection, differences in nuclear uptake of exogenous DNA, and differences in the replication efficiencies of plasmid DNA (Marini *et al*, 1988; Holt *et al*, 1990).

Transfection of blastema mesenchyme using the plasmid pSVluc resulted in consistenly higher levels of enzyme expression as compared to pRSVL. This result is in contrast to that obtained by Holt *et al* (1990). In their study, pRSVL-transfected brains yielded 12 times more luciferase activity than pSV2L *in vivo*. The latter is a SV40 promoter containing construct, similar to pSVluc. There are no published reports comparing the strength of viral or other eukaryotic promoters in newt blastema cells. However, it has been suggested that the SV40 promoter is a strong constitutive promoter in newt blastema cell lines (Brockes, personal communication). Thus, the SV40 promoter may be significantly stronger than the RSV LTR in the newt limb blastema.

Isolated blastema mesenchyme was also incubated with a plasmid containing a gene coding for β-galactosidase in a 1:3 ratio with Lipofectin. No enzyme activity was detected when plasmid DNA was incubated with blastema mesenchyme without the presence of Lipofectin. Incubation with Lipofectin resulted in a low transfection efficiency, with only a few cells staining blue in whole mounts. This is again comparable to a result in *Xenopus* embryonic brain tissue. Only 1 or 2 β-galactosidase positive cells per transfected brain were visible, after lipofection using plasmids containing the lacZ gene and either an elongation factor 1a or an hsp70 promoter (Holt *et al*, 1990).

Holt *et al* (1990) state that the results from the two plasmids with different reporter constructs may not be directly comparable because the threshold for detectability of β-galactosidase may be higher than that for luciferase. However, when the results of the

present *in vivo* study are compared, it is evident that it was possible to detect significantly more β-galactosidase activity than luciferase activity after injection of plasmid DNA into newt blastema mesenchyme.

In vivo injection of pSVluc or pRSVL, either alone or in a 1:3 ratio with lipofectin, resulted in some luciferase expression 4 days later. Again, transfection with pSVluc resulted in a higher level of expression than pRSVL. Luciferase activity was approximately 10-fold less when a DNA-lipofectin mixture (1.6μg DNA) was injected into blastema tissue *in vivo* than when tissue was incubated in DNA-Lipofectin (22.5 μg DNA) *in vitro*. Holt *et al* (1990) reported a 100-fold decrease in luciferase activity when making a similar comparison in *Xenopus*.

As with the *in vitro* results, expression of luciferase activity was also quite variable *in vivo*. In all cases, luciferase expression *in vivo* was very low. Regardless, it is very significant that injection of plasmid DNA without Lipofectin resulted in measurable levels of enzyme activity. When DNA was incubated *in vitro* with blastema mesenchyme without prior complex formation with Lipofectin, luciferase expression was not detected at any time. This is not surprising, however. Both DNA and the cell membrane have a net negative charge, thus it is unlikely that a polynucleotide would associate with a cell membrane and be transported into the cell cytoplasm.

Wolff *et al* (1990) have reported significant levels of luciferase activity after injection of plasmid DNA into mouse quadricep muscle *in vivo*. After injection of 100μg of pRSVL, they

were able to measure 320pg of luciferase activity per muscle. The authors suggest that because of its unique structural features, muscle tissue may be particularly amenable to transfection by foreign polynucleotides. For example, skeletal muscle is composed of multinucleated cells, and contains an elaborate sarcoplasmic reticulum, and transverse tubular system. The latter contains extracellular fluid and penetrates deep into the cell (Wolff *et al*, 1990).

It is interesting that significantly higher levels of β-galactosidase expression were obtained when plasmid DNA was injected alone than when complexed with Lipofectin prior to injection. In the latter case, only a small volume of DNA could be used for injection, and prior dilutions of DNA and Lipofectin separately in Modified Ringer's solution were not possible. Thus, the DNA and Lipofectin may not have complexed, providing no benefit over the use of plasmid DNA alone. When plasmid DNA was used alone, a larger amount could be injected since no Lipofectin was present.

The results obtained by direct *in vivo* injection of phspPTlacZpA into the distal mesenchyme of the newt forelimb are the most impressive. As mentioned previously, as many as 50% of all blastema cells in an area near the injection site expressed β-galactosidase. This degree of transfection efficiency for newt blastema mesenchyme has not been reported elsewhere using any of the known transfection methods except for *in vitro* microinjection. The mechanism of uptake of DNA by blastema cells is unknown. There is a possibility that the injection procedure damages a

significant number of cells. DNA may then associate with cellular debris and ultimaltely be taken up by neighbouring macrophages and other mesenchymal cells.

It has been possible to measure β-galactosidase activity 14 days after injection of phspPTlacZpA *in vivo*. It is not known how this level of stable expression was achieved. Some plasmid DNA may have become stably integrated into the blastema cell genome, or may remain extrachromosomal and continue to be expressed after heat shock. A Southern blot of genomic DNA using a probe specific for a region of the plasmid used for transfection may be used to determine which alternative is the correct one. Alternatively, the protein may be stable in blastema cells over the 14 day period. In any case, the finding that some level of stable expression of foreign DNA in newt blastema is very encouraging. As mentioned previously, it would eventually be desirable to be able to introduce a gene which may have a role in pattern formation into the cells of a regenerating limb. It is important that the gene product continues to be produced in the cell for a long enough period so that any effects on pattern formation could be determined in some manner.

The method of *in vivo* injection of plasmid DNA is an extremely simple procedure, is very inexpensive, and allows discrete areas within the blastema mesenchyme to be targeted, resulting in relatively high transfection and expression efficiencies. It may be possible in the near future to use this method to misexpress a putative pattern formation gene eg. *wnt* (Nusse and Varmus, 1982) in the mesenchyme cells of a newt blastema.

Holt *et al* (1990) performed an experiment in which *Xenopus*

brain tissue was cotransfected with two different plasmids, one containing luciferase as the reporter gene (pRSVL), the other containing chloramphenicol acetyltransferase as the reporter gene (pRSVCAT). Most of the transfected cells coexpressed luciferase and CAT after simultaneous lipofection with the two cDNA containing plasmids. Also, the intensity of the immunostaining suggested that the plasmids were expressed at similar levels. Thus, it may be possible to transfect the cells of a newt blastema by *in vivo* injection using two constructs simultaneously: one containing a reporter gene, the other a gene of interest that may be involved in pattern formation in the newt limb. Expression of the reporter gene at high levels would suggest a similar level of expression of the putative pattern formation gene. Any alteration in the resulting pattern of the regenerate may thus be attributed to expression of the gene product of interest.

CONCLUSIONS

1). It has been possible to use the technique of Lipofection to introduce cDNA into cultured newt blastema mesenchyme. The transfection efficiency was significantly higher than previously achieved using other transfection techniques, with the exception of microinjection.

2). Transfection of a plasmid with a luciferase reporter gene allows quantitation of expression in a blastema both *in vitro* and *in vivo*.

3). The SV40 promoter in the construct pSVluc is a significantly stronger constitutive promoter than the Rous sarcoma virus LTR in blastema cells of the newt.

4). It is possible to obtain measurable amounts of enzyme activity by introducing plasmid DNA into blastema mesenchyme via *in vivo* injection without lipofectin.

5). Targeted injection of uncomplexed phspPTlacZpA DNA (containing an inducible hsp 68 promoter) results in significant levels of localized β-galactosidase expression. The level of expression is variable from cell to cell.

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Carlone, R.L., R.P. Boulianne, K. Marion Vijh, H. Karn, and G.A.D. Fraser. 1992b. Retinoic acid stimulates the synthesis of a novel heat shock protein in the regenerating forelimb of the newt. Submitted to: *Biochem. Cell. Biol.*

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APPENDIX

TABLE 1

RELATIVE LUMINESCENCE FROM SOLUTIONS CONTAINING PURIFIED LUCIFERASE AND THE APPROPRIATE SUBSTRATES

STANDARD NUMBER	pg LUCIFERASE	RELATIVE LUMINESCENCE
1	0.1	0.14
2	1.0	0.27
3	2.0	0.36
4	5.0	0.61
5	10.0	1.16
6	20.0	2.71
7	100.0	18.90

TABLE 2

AMOUNT OF LUCIFERASE EXPRESSION IN HELA CELLS AFTER LIPOFECTION USING A RATIO OF DNA:LIPOFECTIN OF 1:3

RELATIVE LUMINESCENCE (mV)	pg LUCIFERASE	µg PROTEIN	pg/µg PROTEIN
0.26	1.0	0.8	1.25
0.30	1.5	0.8	1.90

TABLE 3

EXPRESSION OF LUCIFERASE ACTIVITY IN NEWT BLASTEMA MESENCHYME FOLLOWING LIPOFECTION WITH VARYING RATIOS OF PLASMID DNA (pSVLUC) TO LIPOFECTIN

RATIO DNA:LIPOFECTIN	RELATIVE LUMINESCENCE (mV)	pg LUCIFERASE	µg PROTEIN	pg/µg PROTEIN
1:0	0.00	0.0	0.0	0.00
1:1	0.30	1.45	24.1	0.06
1:2	0.47	3.40	21.3	0.16
1:2.5	0.85	8.16	20.4	0.40
1:3	1.32	12.2	25.4	0.48
1:3.5	0.80	7.05	23.5	0.30
1:4	0.17	0.25	25.0	0.01

TABLE 4

EXPRESSION OF LUCIFERASE ACTIVITY IN NEWT BLASTEMA MESENCHYME FOLLOWING LIPOFECTION WITH INCREASING AMOUNTS OF pSVLUC PLASMID DNA

AMOUNT OF DNA (μg)	RELATIVE LUMINESCENCE (mV)	pg LUCIFERASE	μg PROTEIN	pg/μg PROTEIN
0.00	0.20	0.40	20.0	0.02
3.75	1.14	9.72	27.0	0.36
7.50	1.13	9.60	20.0	0.48
22.50	2.40	17.10	28.0	0.48
15.0	2.40	17.1	28.0	0.95

TABLE 5

EXPRESSION OF LUCIFERASE ACTIVITY IN NEWT BLASTEMA MESENCHYME FOLLOWING IN VIVO INJECTION OF pSVLUC OR pRSVL (pg luciferase / μg protein)

AMOUNT OF DNA INJECTED (μg)	pSVLUC	pRSVL
0.0	0.0	0.0
1.6	0.5	0.4
5.0	3.5	0.5

TABLE 6

COMPARISON OF EXPRESSION OF LUCIFERASE ACTIVITY IN NEWT BLASTEMAS MESENCHYME FOLLOWING LIPOFECTION IN VIVO (5μg DNA) OR IN VITRO (22.5 μg DNA) USING A 1:3 RATIO OF DNA:LIPOFECTIN (pg LUCIFERASE/μg PROTEIN)

	pSVluc	pRSVL
IN VIVO	1.5	0.60
IN VITRO	12.0	0.10

TABLE 7

PHENOTYPES OF GENES PRESENT IN THE E. COLI STRAIN DH5a

LOCUS	GENE	PHENOTYPE
end	*endA*^{C}	DNA-specific endonuclease
hsd	*hsdRe*	endonuclease R
sup	*supE*	amber suppressor
thi		thiamine biosynthesis
rec	*recA1*	major recombination gene
gyr	*gyrA*	DNA gyrase
rel	*relA*	regulation of RNA synthesis
arg	*argF*	ornithine carbamoyltransferase
lac	*lacZ*	b-galactosidase